

Supporting Information Appendix

Materials and Methods

Oxysterols and other Chemicals. Oxysterols were purchased from either Sigma-Aldrich (St. Louis, MO) or Avanti Polar Lipids, Inc. (Alabaster, AL). 7α , 25-OHC, 7β , 25-OHC, 7β , 27-OHC and 7-keto, 25-OHC were synthesized in house. The stereochemistry at carbon 25 was 25(R) for all 27-OHCs tested. Girard reagent P was purchased from TCI America (Portland, OR). Other chemicals and cholesterol oxidase (from *Streptomyces sp.*) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade solvents were purchased from EMD (Philadelphia, PA).

Recombinant LBD of ROR γ , ROR α , and ROR β . The ligand binding domains (LBD) of human ROR γ (Genbank accession no. NP_005051, 237-497aa), ROR α (Genbank accession no. NP_599022, 304-556aa), and ROR β (Genbank accession no. NP_008845, 201-452aa) were PCR-amplified and cloned into pET24a (Novagen) for expression in bacteria (the resulting recombinant proteins contained the following sequence upstream of the first LBD residue: MAHHHHHHAGGAENLYFQGAMD). After transformation of BL21DE3 GOLD (Agilent), 10 to 20 L cultures were grown to 1 OD and induced with IPTG overnight. Pellets were resuspended in approximately 800 mL of lysis buffer (50 mM HEPES, pH 7.8, 150 mM NaCl, 20mM imidazole, pH8.0, 5% Glycerol, 1 mM TECP) and disrupted with a microfluidizer, two times at 85 psi. Lysates were cleared by centrifugation at 38,400g for 1h followed by IMAC chromatography. ROR α -LBD was further purified by anion exchange chromatography (Q HP, GE Life Sciences).

ROR γ or ROR γ dependent Luciferase Reporter Assays. For expressing the chimeric ROR γ -GAL4 protein, human RORC (ROR γ) LBD (Genbank accession no. NP_005051, aa 258-518) was PCR-amplified from genomic DNA and cloned into a GAL4 DNA Binding Domain (DBD)-containing vector, pBIND (Promega, Madison, WI), using restriction sites BamHI/NotI (Forward Primer: 5'-GAATCCCCGGGGATCCCCAGCACACCGGAGGCACCC-3'; Reverse Primer: 5'-AAGTCCGCGGCCGCTCACTTGGACAGCCCCACAGGTGACTCGG-3'). The insert sequence was confirmed by DNA sequencing (Eton Bioscience, San Diego, CA). The reporter

assay was performed by transiently transfecting HEK293T cells with 5 μ g of pBIND-ROR γ LBD and 5 μ g pGL4.31 (Promega, Madison, WI) using Fugene 6 (Invitrogen, Carlsbad, CA) at a 1:6 ratio of DNA:Fugene6 in a T-75 flask. Twenty-four hours after bulk transfection, cells were plated into 96-well plates at 50,000 cells/well in phenol-red free DMEM containing 5% Lipid Reduced FCS and Pen/Strep. Cells were then treated with compounds for a further 24 hours. Media was removed and cells were lysed with 50 μ L 1x Glo Lysis Buffer (Promega, Madison, WI). Subsequently, 50 μ L/well of Dual Glo Luciferase Reagent s added and, after a 10 minute incubation, chemiluminescence was read on an Envision™ for firefly luciferase signal, the expression of which is ROR γ -GAL4-dependent. Finally, 50uL/well of Stop and Glo reagent (Promega, Madison, WI) was added and after a 10 minute incubation chemiluminescence was read again on Envision for detection of the renilla luciferase signal - renilla luciferase is constitutively expressed and used to normalize the firefly luciferase signal.

For expressing full length ROR γ or ROR γ t proteins, full length ROR γ and ROR γ t cDNA were cloned into a pcDNA4-HisMax vector (Invitrogen). Briefly, PCR was performed on a vector containing full length ROR γ (accession number: U16997) with the following primers, forward: 5'-ATCAGAATTCATGGACAGGGCCCCACA-3'; reverse: 5'-ATCACTCGAGCTACTTGGA CAG-3'. Both the resultant PCR product and pcDNA4-HisMax were digested with EcoR1 and Xho1 and the PCR fragment was subsequently ligated to generate pcDNA4-HisMax-ROR γ . Similarly, full length ROR γ t (accession number: NM_001001523) was obtained through PCR with the following primers: forward: 5'-ACGAATAAGAATCCATGAGAACACAAT-3' and reverse: 5'-AAGTCCGCGGCCGCTCACTTGGACAGC-3' followed by cloning into the EcoR1 and Not1 sites of pcDNA4-HisMax. The reporter assay system was comprised of HEK293T cells transiently transfected with pcDNA4-HisMax-ROR γ or the pcDNA4-HisMax-ROR γ t, a gene reporter construct which contains a CNS2 element fused to the il17 promoter upstream of a luciferase gene (pGL4.25-CNS2-IL17p), and pBIND vector (Promega). The il17 promoter (-1248 bp upstream) and CNS2 domain (aka CNS5) were cloned from Jurkat genomic DNA by PCR with il17 primers 5'-CACTCGAGCTCATCTGTCCTCCAATTC-3' and 5'-CAAA GCTTCGTTGTTTCTTCCAATCAACT-3' and CNS2 forward and reverse primers, 5'-GTAGCTAGCTAGCAACCTCTATATTGCCT-3' and 5'-GTACTCGAGCTTCCCTTTGGTA

ATGCTG-3', respectively. Cell culture and measurement of ROR γ - or ROR γ t-dependent firefly or constitutively expressed renilla luciferase activities was the same as described above.

Nuclear Receptor Reporter Assays for Selectivity. Cell-based luciferase reporter assay kits for CAR, ER α , ER β , FXR, GR, PPAR α , PPAR δ and PPAR γ were purchased from Indigo Biosciences, Inc. (State College, PA). Cell-based fluorescence reporter assay kits for LXR α and LXR β were purchased from Invitrogen (Carlsbad, CA). Each kit contained their respective agonists as positive controls and the reporter assays were performed according to the manufacturer's instructions. Oxysterols were assessed in dose-response experiments in 8-point, 1:3 serial dilutions starting at 30 μ M final concentration. Compound serial dilutions were prepared in 100% DMSO and transferred into compound screening media. Briefly, for Indigo Biosciences assays, 100 μ l of reporter cell suspension was dispensed into 96-well assay plates and 100 μ l of test compound was added to the respective wells in triplicates. Plates were transferred into a 37°C, humidified 5% CO₂ incubator for 23 hours. 100 μ l of Luciferase Detection Reagent was added to each well of the assay plate, incubated for 15 minutes at room temperature and luminescence was quantified using SoftMax Pro 4.7.1 on Luminometer LMaxII 384 from Molecular Devices. For LXR α and LXR β assays, 32 μ l of reporter cell suspension was dispensed into 384-well assay plates and 8 μ l of test compound was added to the respective wells in triplicates. Plates were transferred into a 37°C, humidified 5% CO₂ incubator for 16 hours. 8 μ l of LiveBLAzer™-FRET B/G Substrate was added to each well of the assay plate for 2 hours at room temperature and fluorescence emission values at 460 nm and 530 nm were obtained using Wallac EnVision Manager 1.12 on Perkin Elmer's EnVision 2104 Multilabel Reader. % Response and % Inhibition values were calculated and IC₅₀/EC₅₀ values were generated using GraphPad Prism 5 for Windows, Version 5.01.

³H-25-OH/ROR γ LBD SPA Binding Assay. ³H-labeled 25-OHC was purchased from Perkin Elmer (specific activity: 80 Ci/mmol). Briefly, a homogenous SPA binding assay was assembled in non-binding surface 96-well plates (Corning #3990) by adding purified recombinant ROR γ LBD-HIS protein (500ng/well), ³H-labeled 25-OHC (50nM) and titrated concentrations of oxysterols (first, the serial dilution was performed in DMSO, and then compounds were diluted 1:10 in assay buffer before adding a 1:10 volume to the final binding reaction) into 100 μ l final

volume of assay buffer (50mM Hepes, 0.01% BSA, 150mM NaCl, 5mM MgCl₂, 10% glycerol, 1mM DTT, and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN)). The binding plate was read on Topcount following 1h incubation at room temperature. Percent of inhibition was calculated based on “without ROR γ LBD-HIS protein” well as 100% inhibition and “DMSO only” well as no inhibition. Percent inhibition values were plotted vs. compound concentration and IC₅₀ were calculated using GraphPad Prism 5. K_i values were calculated based on $K_i = IC_{50} / ([^3H\text{-}25\text{-OHC}] / K_d + 1) = IC_{50} / (50nM / 10nM + 1) = IC_{50} / 6$.

ROR γ LBD Surface Plasmon Resonance (SPR) Binding Assay. Biacore studies were performed by Biosensor Tools (Salt Lake City, UT) on a Biacore S51. About 8000 RU of streptavidin (Pierce Biotechnology Inc, Rockford, IL) was amine coupled to a CM5 sensor chip using standard NHS/EDC (0.1 M/0.4 M) activation. Streptavidin was injected in 10 mM NaAcetate, pH 4.2 at 10 μ g/mL for seven minutes. Surfaces were blocked with 1 M ethanolamine for 3 minutes. The running buffer for immobilization included 10 mM HEPES, pH 7.4, 150 mM NaCl. Coupling was done with the sensor chip equilibrated at 30°C. Biotinylated peptides NCORNR (CDPASNLGLEDIIRKALMGSFDDK) and NCOA3 (CSLLQEKHRIL HKLLQNGNS) were prepared at a concentration of 100 nM and injected over spots 1 and 2 within the same flow cell. NCORNR captured to ~400 RU and NCOA3 captured to ~1500 RU (green trace). The running buffer for the ROR γ t/compound studies contained 10 mM HEPES, pH 7.4, 150 mM NaCl, 2.5 mM DTT, 5% glycerol and 3% DMSO. The binding data for ROR γ t was collected at 25°C. Surfaces were regenerated with a 7 second injection of 0.25% SDS prepared in the running buffer. Oxysterols were serially diluted in 100% DMSO and then incubated with a constant concentration of the ROR γ t (1 μ M) using 10 μ M as the highest compound concentration.

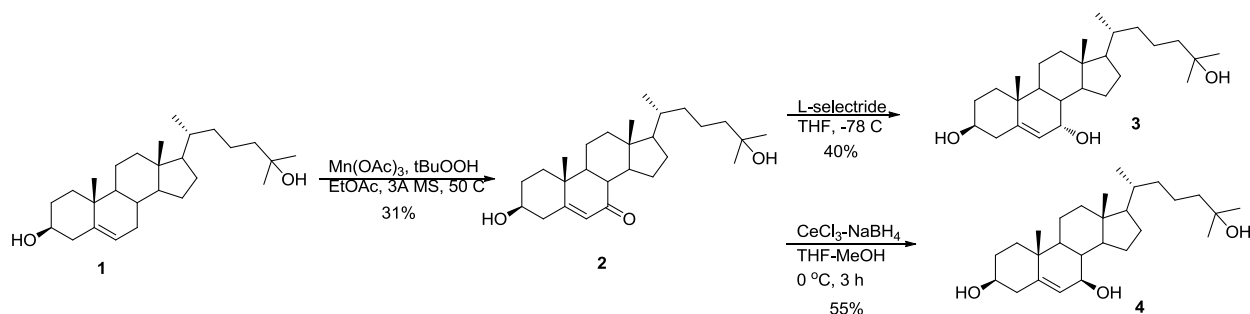
ThermoFluor Binding Assay. In a ThermoFluor[®] experiment (2-4), where protein stability is monitored as the temperature is steadily increased, an equilibrium binding ligand causes the midpoint of an unfolding transition (T_m) to occur at a higher temperature. The shift in the melting point described as a ΔT_m is proportional to the concentration and affinity of the ligand. The compound potency may be compared as a rank order of either ΔT_m values at a single compound concentration or in terms of K_D values, estimated from concentration response curves.

ThermoFluor[®] experiments were carried out using instruments owned by Janssen Research & Development, L.L.C. 1,8-ANS (Invitrogen:A-47) was used as a fluorescent dye. Protein and compound solutions were dispensed into black 384-well polypropylene PCR microplates (Abgene: TF-0384/k) and overlaid with silicone oil (1 μ L, Fluka, type DC 200: 85411) to prevent evaporation.

Assay plates were robotically loaded onto a thermostatically controlled PCR-type thermal block and then heated at a typical ramp-rate of 1°C/min for all experiments. Fluorescence was measured by continuous illumination with UV light (Hamamatsu LC6) supplied via fiber optic and filtered through a band-pass filter (380-400 nm; >6 OD cutoff). Fluorescence emission of the entire 384-well plate was detected by measuring light intensity using a CCD camera (Sensys, Roper Scientific) filtered to detect 500 ± 25 nm, resulting in simultaneous and independent readings of all 384 wells. Reference wells contained recombinant LBD of ROR γ t, ROR α or ROR β , and the assay conditions for the proteins were as follows: ROR γ t - 0.065 mg/mL of protein, 60 μ M 1,8-ANS, 100 mM HEPES pH 7.0, 10 mM NaCl, 2.5 mM GSH, 0.002% Tween-20; ROR α - 0.1 mg/mL of protein, 70 μ M 1,8-ANS, 25 mM HEPES pH 7.0, 50 mM NaCl, 0.001% Tween-20; ROR β - 0.08 mg/mL of protein, 60 μ M 1,8-ANS, 50 mM HEPES pH 7.0, 100 mM NaCl, 0.001% Tween-20. All 3 assays were performed in the absence and in the presence of 100 μ M co-activator peptide SRC1 (LTERHKILHRLQLQEGSPSD, England Peptide custom synthesis).

Compounds were arranged in a pre-dosed mother plate (Greiner Bio-one: 781280), wherein compounds were serially diluted in 100% DMSO. Following compound dispense of 50 nL directly into assay plates, protein and dye in buffer was added to achieve the final assay volume of 3 μ L, followed by 1 μ L of silicone oil. The binding affinity of compounds tested was estimated as described previously (4).

Synthesis of 7 α / β /keto, 25-OHC. 7-keto, 25-OHC was prepared from commercially available 25-OHC according to the literature method (5). 7 α , 25-OHC and 7 β , 25-OHC were both prepared from 7-keto, 25-OHC by stereoselective reductions (6, 7). The resulting compounds were confirmed by MS and NMR analysis.



7-keto-dihydroxylcholesterol (2): To a solution of 25-hydroxycholesterol (**1**, 10 g, 24.8 mmol, 1.0 equiv.) in EtOAc (100 mL), was added *tert*-butyl hydroperoxide in decane (5-6 mol/L, 29.8 mL, 6 equiv.) and 3A molecular sieves (14 g). The mixture was stirred at room temperature under N_2 for 30 min. $\text{Mn}(\text{OAc})_3 \cdot 2\text{H}_2\text{O}$ (576 mg, 2.48 mmol, 0.1 equiv.) was added under N_2 and the mixture was stirred at 50 °C for 16 h. The insoluble solid was filtered off and washed with EtOAc. The filtrate was concentrated and directly loaded on a silica gel column for chromatographic purification to afford the title compound (3.2 g, 7.7 mmol, 31%). Spectroscopic data matched previously reported data (1).

7 α -25-dihydroxylcholesterol (3): To a solution of compound **2** (3.0 g, 7.2 mmol, 1.0 equiv.) in THF (50 mL) at -78 °C was added L-Selectride (1.0 mol/L in THF, 25.2 mL, 3.5 equiv.). After stirring at -78 °C for 3 h, AcOH (3 mL) was added to quench the reaction and the reaction mixture was then warmed to room temperature. Water (200 mL) and EtOAc (200 mL) were added and the suspension was stirred at room temperature for 16 h. The insoluble white solid was collected by filtration, washed with EtOAc, dried to afford the title compound (0.8 g). The organic layer of the mother liquor was separated and concentrated. The residue was recrystallized from hot EtOAc to afford another 0.4 g of the pure title compound. The combined yield was 40%. Spectroscopic data matched previously reported data (1).

7 β -25-dihydroxylcholesterol (4): Compound **2** (50 mg, 0.12 mmol, 1.0 equiv.) was dissolved in a mixed mixture of THF-MeOH (10 mL + 5 mL). $\text{CeCl}_3 \cdot 7\text{H}_2\text{O}$ (134 mg, 0.36 mmol, 3.0 equiv.) was added followed by NaBH_4 (13.7 mg, 0.36 mmol, 3.0 equiv.). After stirring at room temperature for 3 h, 1 mol/L HCl (2 mL) was added to quench the reaction. The reaction mixture was partitioned between water and EtOAc. The organic layer was concentrated and the crude

product was purified on silica gel normal phase column chromatography to afford the title compound (27 mg, 0.06 mmol, 55%). Spectroscopic data matched previously reported data (1).

The same two step sequence was used to prepare 7 β -27-dihydroxylcholesterol in 12% overall yield, starting from commercially available 27-hydroxycholesterol.

Extraction of Lipids from Mouse Spleen. Frozen mouse spleens (~ 60-80 mg wet weight) were weighted and homogenized with 0.3 mL PBS buffer with a Handishear homogenizer, and the total lipids were extracted by Bligh-Dyer method (8). Briefly, 1.5 mL of CHCl₃/ MeOH (1:2 v/v) was added to form the azeotrope condition for lipid extraction. The samples were centrifuged for 5 min at 3000 rpm to pellet insoluble material and the supernatant was collected. Deuterated surrogate sterol standards (40ng per standard per spleen sample) were spiked to the supernatant, followed by 0.5 mL of CHCl₃ and 0.5 mL of PBS buffer to form bi-layers. After centrifugation briefly at 3000 rpm, the resulting organic phase (lower layer) was collected and dried with Genevac.

Cholesterol Removal by Solid Phase Extraction. The removal of cholesterol was done according to the method developed by Wang and Griffiths (9). Each of the above dried lipid extract was dissolved in 3 mL of (70% ethanol, 30% water), and loaded to a C18 SPE cartridge (Waters Sep-Pak Vac 3cc 200 mg) which has been pre-washed with 6 mL of 100% ethanol followed by 6 mL of (70% ethanol, 30% water) for equilibrium. The flow-through was collected into a clean glass tube. The SPE cartridge was further washed with 6 mL of (70% ethanol, 30% water) and the eluent was collected into the same tube and subsequently dried with Genevac.

Enzymatic Oxidation and Girard Reagent P Derivatization of Oxysterols. The derivatization of oxysterols was done according to the method developed by Wang and Griffiths (9) with modifications. To the above dried sample, 200 μ L isopropanol was added. After vortex, 2 mL 50 mM KH₂PO₄ buffer (pH 7.0) was added followed by 5 min sonication. 1 Unit of cholesterol oxidase (10 μ L) was then added and the sample was incubated in a 37 °C water bath for 1 h and then dried by Genevac overnight.

To the above dried sample, 100 μ L water and 100 μ L isopropanol were added to dissolve the solid with sonication. A freshly prepared GP solution (1 mL methanol, 72 mg of GP, 50 μ L of

acetic acid, sonicated to a clear solution) was added to the sample. This induced some precipitation, but after sonication for 10 min the solution appeared to be uniform. The sample was covered with aluminum foil and shaken at 500 rpm at room temperature overnight.

The GP reacted sample was dried by Genevac, and 2 mL water was added to dissolve the solid. The sample was then extracted with 3x 2 mL ethylacetate (EA). The combined EA phase was dried by Genevac, dissolved in 1 mL methanol, filtered through a spin-filter (Millipore Ultrafree PVDF 0.22 μ m), transferred to an HPLC vial, dried by Genevac again, re-suspended in 100 μ L methanol and subjected to LC/MS/MS analysis.

Quantification of Oxysterols with LC/MS/MS. An aliquot of 5 μ L of sample was injected on the Acquity ultra performance liquid chromatography (UPLC, Waters, Milford, MA). Oxysterols were separated on an ACE 3 C18-AR column (150 mm x 2.1 mm, MAC-MOD, Chadds Ford, PA) using a flow rate of 300 μ L/min at 40 °C. The oxysterols were eluted using a stepwise gradient with mobile phase A consisting of H₂O-MeOH-formic acid (70:30:0.1, v/v/v) and mobile phase B consisting of MeOH-formic acid (100:0.1, v/v). The gradient program employed was: 0-1.0 min 80% A, 3.5 min 40% A, 8.5 min 40% A, 11.5 min 20% A, 16.5 min 20% A, 20.0 min 10% A, 22.5 min 10% A, from 25.0 to 30.0 min, 80% A. The gradient was linear between each step.

The mass spectrometric detection was operated on an AB SCIEX API 4000 system (AB SCIEX, Foster City, CA) equipped with a Turbo Ionspray source. The data acquisition software used to operate the mass spectrometry was Analyst version 1.5. Quantitation was performed by scheduled multiple reaction monitoring (sMRM) in positive mode with parameters as follows: 4500 V ion spray voltage; 20 psi curtain gas; 30 psi ion source gas 1 (GS1); 30 psi ion source gas 2 (GS2); 6 psi collision gas (CAD); 10 V entrance potential (EP); 10V collision cell exit potential (CXP); 500 °C and 60 s MRM detection window. The optimized MRM fragmentation transitions and parameters for declustering potential (DP) and collision energy (CE) for each lipid are listed in **Table S3**. Representative extracted ion chromatogram of LC-MS/MS analysis of standard solutions of oxysterols and cholesterol was shown in **Figure S15**.

Quantitation was performed using a stable isotope dilute technique with the signature transition of each oxysterol versus internal standard monitored on a 8-point calibration curve. Data analysis was done in Multiquant 2.0 (AB SCIEX, Foster City, CA). Standard curves were generated by mixing the standard stocks with the dilution solution (0.15 mg/ml cholesterol, 0.5 mg/ml BSA, 1 mg/ml Na₂S₂O₅ in 30% water, 70% EtOH) at 7 final concentrations (0.03, 0.09, 0.27, 0.81, 2.43, 7.29, 21.87 ng). 10 μ L of the deuterated internal standard stock (1 ng/ μ L) was spiked to each standard curve samples and spleen samples. The extraction and derivatization steps described previously (1) were carried out in parallel for both standard curve and spleen samples. The standards mixes were brought up to 100 μ L with MeOH for LC/MS/MS quantification.

Mice. Ch25h KO mice on a C57BL/6J background, Cyp27a1 KO mice and ROR γ t KO mice on a mixed C57BL/6J and C57BL/6N genetic background, and C57BL/6J control mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). All studies in mice have been carried out in accordance with the animal use guidelines and approved ICAUC protocols by Janssen R&D. LLC.

Antibodies and Flow cytometry. The following antibodies (anti-mouse or anti-human) were purchased from BD Biosciences (San Diego, CA): anti-CD4-allophycocyanin, anti-CD4-PerCp, anti-CD4-FITC, anti-CD44-PE, anti- $\gamma\delta$ T cells receptor-FITC, anti-CD62L-FITC, anti-CD45RO-allophycocyanin and anti-CCR6-PE. Human or mouse anti-IFN γ , anti-TNF- α , anti-IL-17A, and anti-IL-22 (all PE or allophycocyanin conjugated) were purchased from eBioscience (San Diego, CA). To detect intracellular cytokines, cells were re-stimulated with leukocytes activation cocktail (BD Biosciences) in the presence of monensin (eBioscience) for 4 hours. Reagents for cell fixation and permeabilization for detecting intracellular cytokines were obtained from eBioscience, and staining was performed according to the manufacturer's instructions. Cells were examined by flow cytometry using the FACSCalibur or FACSCanto II (BD) and analyzed with FlowJo software (Tree Star, San Carlos, CA).

Mouse Th17 Cell Activation and Differentiation in vitro. Total or naïve CD4⁺ T cells were isolated from splenocytes of C57BL/6J WT or various KO mice using CD4 T cell isolation kit II or naïve CD4 T cells isolation kit II, respectively, by following manufacturer's instructions

(Miltenyi Biotec, Auburn, CA). Cells were resuspended in medium of RPMI-1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, glutamate, and β -mercaptoethanol and were added to 96-well plates at 2×10^5 cells per 100 μ L per well. 50 μ L of oxysterols or/and ursolic acid were added into each well at final DMSO concentration of 0.2%. Cells were incubated for 1h, and then 50 μ L of Th17 cell differentiation medium was added to each well. The final concentrations of antibodies and cytokines (R&D Systems, Minneapolis, MN) in differentiation medium were: 1 μ g/mL anti-CD3, 5 μ g/mL anti-CD28 (BD Pharmingen, San Diego, CA), 10 μ g/mL anti-IL-4, 10 μ g/mL anti-IFN γ , 10ng/mL IL-23, 10ng/mL IL-6, and 10ng/mL TGF β . Cells were cultured at 37°C and 5% CO₂ for 2-6 days. Cells were collected on specified days and stained for intracellular cytokines.

Gene Expression Studies in Mouse Splenocytes or Th17 Cells. Total RNA was extracted from mouse splenocytes or T cells using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA was quantified using NanoDrop 1000 (Thermo Scientific). Real-time RT-PCR was carried out using the commercial Taqman RNA-to-Ct 1 step kit (Life Technologies) in a 50 μ L reaction mixture containing 1.25 μ L of 40 \times Taqman RT Enzyme mix, 25 μ L of 2 \times Taqman RT-PCR Mix, 11.25 μ L RNase free dH₂O, 10 μ L RNA sample and 2.5 μ L of Taqman Gene Expression Assay or in the case of the ROR γ t custom primers set 0.075 μ L of each oligo and 0.0625 μ L of the probe were used. Each reaction had a final concentration of 100ng of RNA and 0.25 μ M of Taqman Gene expression assay or ROR γ t primers set. The real-time reaction was conducted on the 7500 real-time PCR System (Applied Biosystems by Life Technologies). The reactions underwent 15 min at 48°C, 10 min at 95°C, then 40 cycles of 15s at 95°C and 60s at 60°C. The levels of mRNA expression for each gene were normalized to beta-2 microglobulin expression level and calculated automatically by the SDS software (Version 1.2.2; Applied Biosystems by Life Technologies) using the comparative CT method ($\Delta\Delta$ CT), and the values were expressed as relative arbitrary units. The results were graphed and statistical analysis done with GraphPad Prism 5 using 2-way ANOVA.

Taqman Gene expression assays (Life technologies) were as follow: B2M (ID Mm00437762_m1), IL-17A (ID Mm0043918_m1), IL-17F (ID Mm00521423_m1), IL-22 (ID Mm00444241_m1), IFN γ (ID Mm00801778_m), ROR alpha (ID Mm1173766_m1), RORC (ID

Mm01261022_m1). ROR γ t-specific primers were custom made with sequence as follow: forward 5'- AGGACAGGGAGCCAAGTTCTC-3', probe: 5'-TGAGAACACAAATTGAAG-3' and reverse: 5'-TGTCCCCACAGATCTTGCAA-3'.

Conversion of ^3H -7 α -OHC, ^3H -7 β -OHC or ^3H -27-OHC by Mouse Th1 or Th17 Cells in Culture. Total or naïve CD4⁺ T cells isolated from splenocytes of C57BL/6J or Cyp27a1 KO mice (female, 6-8 weeks) were cultured under Th17 differentiation condition as described above or under Th1 differentiation condition for 3 or 6 days. The Th1 differentiation medium contained 1 $\mu\text{g}/\text{mL}$ anti-CD3, 5 $\mu\text{g}/\text{mL}$ anti-CD28, 10 $\mu\text{g}/\text{mL}$ anti-IL-4, and 10 ng/mL IL-12. ^3H -7 α -OHC or 7 β -OHC (American Radiolabeled chemicals, ART 1739 or 1740) or ^3H -27-OHC (synthesized by Quotient Bioresearch, UK) was then added, and cell culture medium was collected after 8 hr and 24 hr. Cell culture medium was analyzed by HPLC as previously described³. Briefly, 0.8 mL samples of conditioned culture medium were frozen and completely lyophilized. Each sample was then reconstituted with 0.5 ml of 5 mM NH₄OAc in 85% methanol (mobile phase A), mixed, sonicated for 5 minutes, and then centrifuged for 5 minutes at 20,000g (room temperature). The supernatant was transferred to a clear glass HPLC vial. 0.25 mL of each sample was injected onto an Agilent Eclipse XDB-C18 (5 μm , 4.6 x 250 mm) column running at 0.75 mL/min. After injection the sample was eluted in a 1% methanol/minute gradient to 5 mM NH₄OAc in 100% methanol. One minute fractions were collected. In some cases 0.5 min fractions were collected (see figure legends). A 50 μL sample of each fraction was transferred to a 96-well GF/C plate and dried at 60°C for 10 minutes. 50 μL of Microscint 40 (Perkin Elmer, Boston MA) was added to each well and radioactivity was measured in a Top Count (Perkin Elmer, Boston, MA). For LCMS of tritiated and control hydroxycholesterols the same column and HPLC conditions were used. An Agilent 1100 LC/MSD was used to collect data, which was analyzed with Agilent Chemstation software.

Conversion of ^3H -7 α -OHC or ^3H -7 β -OHC to Dihydroxycholesterols by COS7 Cells Transiently Expressing Recombinant Human CYP27A1 Protein. Human CYP27A1 (Genbank Accession # NM_000784.3) was PCR amplified using human liver cDNA as the template. The PCR primers used for human CYP27A1 are listed as follows. Forward primer: 5' acgtcaGAATTCgccaccATGGCTGCGCTGGGCTGCGCGAGGCTGA 3', Reverse Primer: 5'

ACGTCAGcgggccgcTCAGCACTGTCTCTGCAGGAACTGCAG 3' The PCR conditions were 94°C 20 sec, 65°C, 20 sec, and 72°C 4 min for 35 cycles. The cDNA was cloned into a mammalian expression vector pcDNA3.1+ (Invitrogen, Carlsbad, CA) and the insert sequenced to confirm the identity. COS7 cells grown in 6-well dish were either transiently transfected with 2µg CYP27A1 construct with Lipofectamine or mock transfected with empty vector. 24h post-transfection, media was changed to 2ml Opti-MEM I containing 1% FAF BSA (1X sodium pyruvate, 1X pen/strep) and incubated overnight. Next day, cells were spiked with 5ul (5uCi) ³H-7α-OHC (American Radiolabeled Chemicals, Cat # ART 1739) or ³H-7β-OHC (American Radiolabeled Chemicals, Cat # ART 1740). Cells were incubated for 8 hours and the media was collected in conical tube. The collected media was freeze dried and analyzed by HPLC as described above.

Human Th17 Cell Activation and Differentiation in vitro. Total or naïve CD4⁺ T cells were isolated from peripheral blood mononuclear cells (PBMC) of healthy donor using CD4 T cell isolation kit II or naïve CD4 T cells isolation kit II respectively, by following manufacture's instruction (Miltenyi Biotec, Auburn, CA). Purity of naïve CD4⁺ T cells were examined using FACS and showed to be >98%. Cells were resuspended in medium of RPMI-1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, glutamate, and β-mercaptoethanol and were added to 96-well plates at 1.5×10^5 cells per 100µL per well. 50µL of oxysterols or/and ursolic acid were added into each well at final DMSO concentration of 0.2%. Cells were incubated for 1h, then 50µL of Th17 cell differentiation medium was added to each well. The final concentrations of antibodies and cytokines (R&D Systems, Minneapolis, MN) in differentiation medium were: 3×10^6 /mL anti-CD3/CD28 beads (prepared using human T cell activation/expansion kit, Miltenyi Biotec, Auburn, CA), 10µg/mL anti-IL-4, 10µg/mL anti-IFNγ, 10ng/mL IL-1β, 10ng/mL IL-23, 50ng/mL IL-6, 3ng/mL TGFβ and 20U/mL IL-2. Cells were cultured at 37°C and 5% CO₂ for up to 10 days. Cells were collected on specified days and stained for intracellular cytokines. Oxysterols in general showed cytotoxicity at concentrations above 1µM for human CD4⁺ T cells in culture.

In vivo Generation of Antigen-specific Th17 Cells and dosing of 7β, 27-OHC For induction of Th17 cells *in vivo*, mice were immunized subcutaneously on day 0 with 100 µg chicken

Ovalbumin (OVA grade-V, Sigma) emulsified in complete Freund's adjuvant (CFA, Chondrex, Redmond, WA) supplemented with 200 mg/mL *Mycobacterium tuberculosis*. After 10 days, draining lymph nodes (DLN) were harvested from immunized mice and the total cells from DLN were re-stimulated with OVA (50 µg/mL) in completed RPMI. After 2 and 6 days, supernatant were collected for ELISA and cells were subjected to intracellular staining for indicated cytokines.

In vivo dosing of 7β, 27-OHC C57BL/6J mice at 11 weeks (n=3 per group) were dosed with 7β, 27-OHC. The compound was dissolved in 20% HPCD, and dosed as solution at 60mg/kg, subcutaneously, twice a day, for a period of 3 days on the lower back. On day 4, the animals were then euthanized according to the IACUC approved protocol, and inguinal lymph nodes were collected. LN cells were activated PMA/inomycin for 4h prior to intracellular staining.

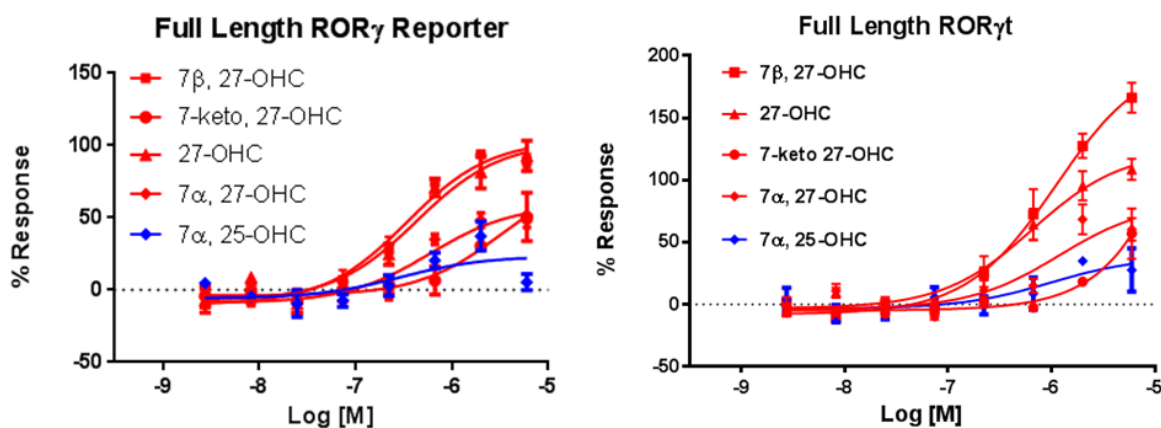
For the OVA/CFA immunization study, one day prior to immunization, female C57BL/6J (n=3 per group) mice were dosed with either vehicle 20% HPCD both by intraperitoneal injection and subcutaneously, UA (ursolic acid) at 150mg/kg in 20% HPCD by i.p, or UA at 150mg/kg by i.p and 7β, 27-OHC at 60mg/kg s.q, bid. The following day the animals were immunized with OVA/CFA (OVA was prepared at 2mg/ml in Saline, added to CFA in 1:1 ratio, the mixture was emulsified using the Handishear homogenizer) and dosed at 100µg/100µl. The dose regiment was repeated as following, 7β, 27-OHC was dosed s.q bid, UA was dosed q.d every other day, and the vehicle group was dosed accordingly. On day 7 post OVA/CFA immunization, the animals were euthanized according to the IACUC approved protocol, and draining inguinal lymph nodes were collected. The total cells from dLN were re-stimulated with OVA (50 µg/mL) in completed RPMI. After 2, supernatant were collected for ELISA and cells were subjected to intracellular staining for indicated cytokines.

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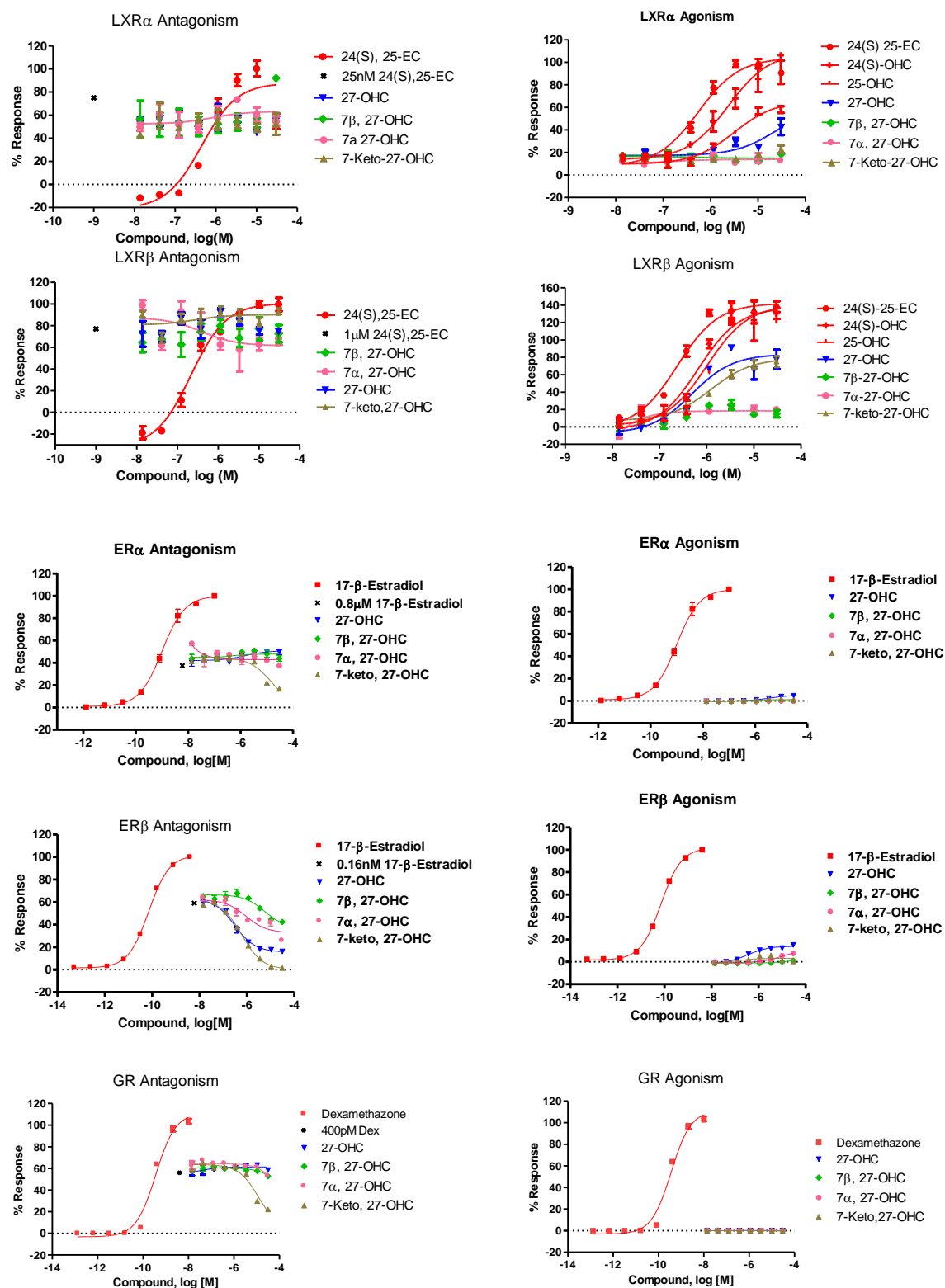
Supporting Information Figures and Tables

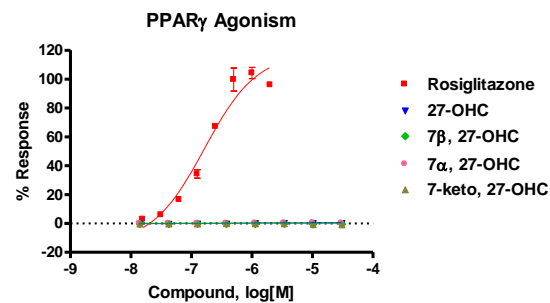
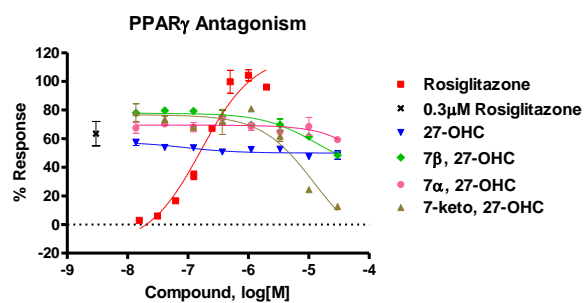
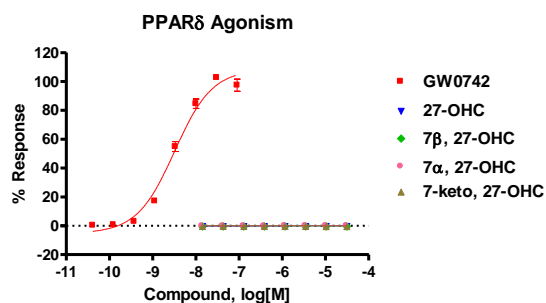
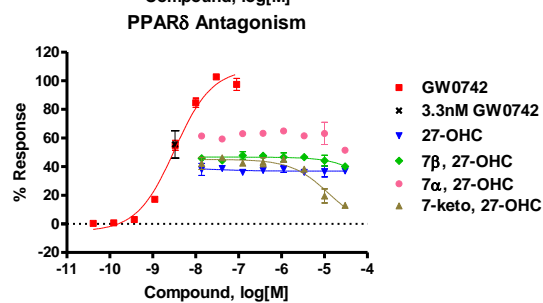
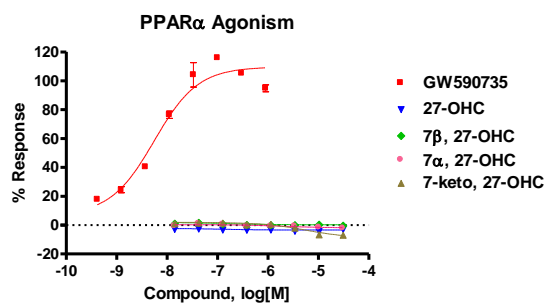
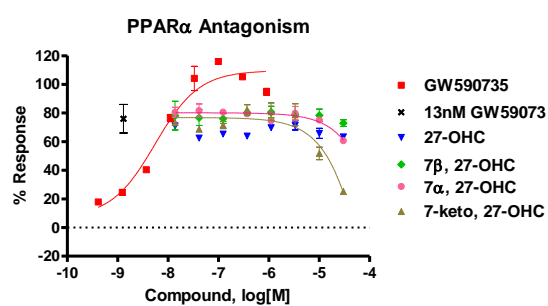
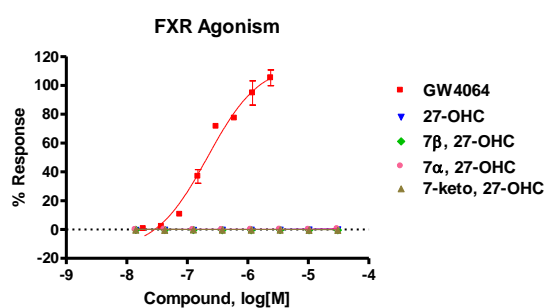
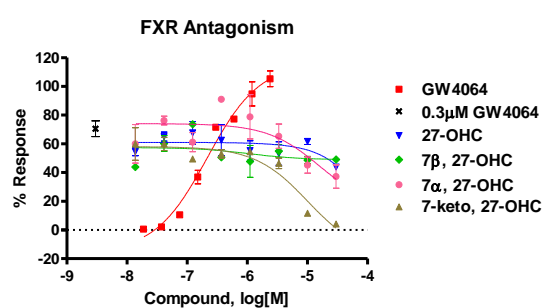
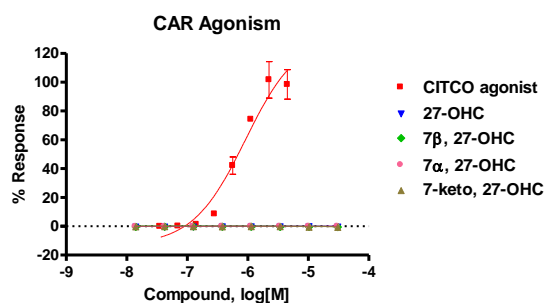
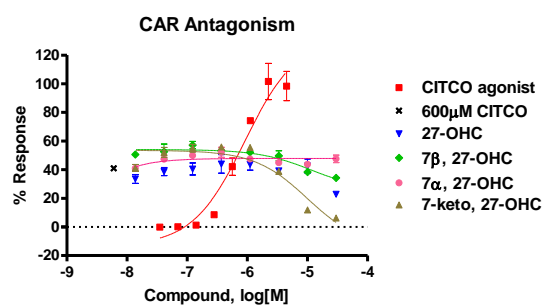
Figure S1. Agonist activities of oxysterols in cell-based full length ROR γ - or ROR γ t-dependent reporter assays. Shown on the top are dose titration curves from representative experiments. For the reporter assay, oxysterols (starting at 6 μ M, 1:3 serial dilutions) were tested in duplicates in the presence (0% of response) or the absence (100% of response) of 1 μ M ursolic acid. EC₅₀ values were calculated using GraphPad Prism 5. Average \pm SD (n = or > 3) of EC₅₀ and efficacy values at the highest tested concentration are shown in the table.



Oxysterols	Cell-based Reporter Assays			
	Full Length ROR γ		Full Length ROR γ t	
	EC ₅₀ (Ave \pm SD, nM)	Efficacy at 6 μ M (Ave \pm SD, %)	EC ₅₀ (Ave \pm SD, nM)	Efficacy at 6 μ M (Ave \pm SD, %)
7 β , 27-OHC	691 \pm 327	89 \pm 17	1045 \pm 141	137 \pm 35
7-keto, 27-OHC	~6,000, >6,000	29 \pm 25	~6,000, >6,000	30 \pm 40
27-OHC	805 \pm 375	84 \pm 12	839 \pm 199	98 \pm 17
7 α , 27-OHC	718 \pm 202	39 \pm 11	970 \pm 338	49 \pm 11
7 α , 25-OHC	>6,000	5 \pm 6	>6,000	15 \pm 11
Cholesterol	>6,000	15 \pm 12	>6,000	15 \pm 7

Figure S2. Nuclear receptor selectivity of oxysterols

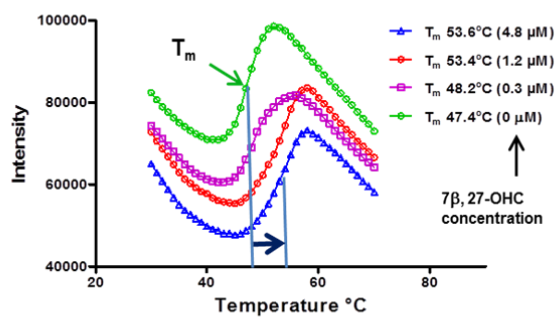




Cell-based nuclear receptor assays were performed according to manufacturer's instructions. Titrated control agonists or oxysterols were tested for agonist mode testing; EC_{50} - EC_{80} of respective control agonists were added to all wells for antagonist mode testing. Titrated concentrations of oxysterols (starting concentration at 30 μ M, 1:3 serial dilutions) were tested in duplicates or triplicates. Background luciferase activity in the absence of a control agonist was defined as 0% response; and maximum luciferase activity induced by a control agonist as 100% response.

Figure S3. Direct binding of selected oxysterols to ROR LBD. a. Thermofluor binding assay with LBDs of ROR γ , ROR α , or ROR β . Representative ThermoFluor melting curves of ROR γ LBD and titrated concentrations (in parenthesis) of 7 β , 27-OHC, and observed melting T_m , which were used to calculate binding K_d . K_d values shown in the table are average values from at least two independent experiments in the presence of SRC1 (w/ Src) or absence (apo). **b.** Determination of K_d value of ^3H -25-OHC binding to ROR γ LBD in a saturation binding assay. Shown in the table are average K_i and efficacy values from 3-6 experiments from the ^3H -25-OHC/ROR γ LBD competitive binding assay.

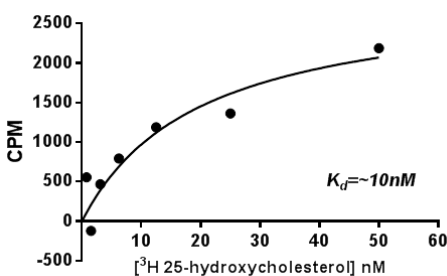
a.



Oxysterol	TF K_d , Average \pm SD, nM, n = 2-3			
	ROR γ apo	ROR γ w/ Src	ROR α w/ Src	ROR β w/ Src
7 β , 27-OHC	28 \pm 3	42 \pm 11	>19,000	40 \pm 11
7-keto, 27-OHC	80 \pm 10	68 \pm 28	3 \pm 1	125 \pm 97
7 α , 27-OHC	450 \pm 166	190 \pm 64	>19,000	9,100 \pm 7,800
27-OHC	85 \pm 58	1,100 \pm 383	>6,000	450 \pm 495
7 α , 25-OHC	320 \pm 174	245 \pm 97	210 \pm 117	>6,000
Cholesterol	>50,000	>76,000	>76,000	75,000

b.

Specific Binding of 25-hydroxycholesterol to bacterially-expressed hROR γ -LBD



Oxysterols	K_i (Ave \pm SD, nM)	Inhibition at 30 μ M (Ave \pm SD, %)
7 β , 27-OHC	120 \pm 110	79 \pm 12
7 α , 27-OHC	1,008 \pm 587	82 \pm 11
25-OHC	280 \pm 180	100 \pm 0

Figure S4. 7β , 27-OHC and 7α , 27-OHC but not 7α , 25-OHC promoted IL-17 production by mouse and human Th17 cells *in vitro*. **a.** Effect of 7β , 27-OHC and 7α , 27-OHC on mouse total $CD4^+$ T cells from wild type (WT) mice activated under Th17 polarizing condition for 3 days in culture. Bar graph showed average \pm SEM from >3 independent experiments. **b.** Effect of 7β , 27-OHC and 7α , 27-OHC on human naïve $CD4^+$ T cells ($CD45RO^-$, $CCR6^-$) activated under Th17 polarizing condition for 10 days in culture. DMSO (vehicle) or 1 μ M ursolic acid (UA) was added 2 h before cell stimulation. Oxysterols (6 μ M for mouse T cells, and 0.3 μ M for human T cells) were added at the start of culture. Bar graph showed average \pm SEM from 5 independent experiments. Statistics by Student's t-test, two-tailed, unpaired, * $p < 0.05$, **, $p < 0.01$, and *** $p < 0.001$.

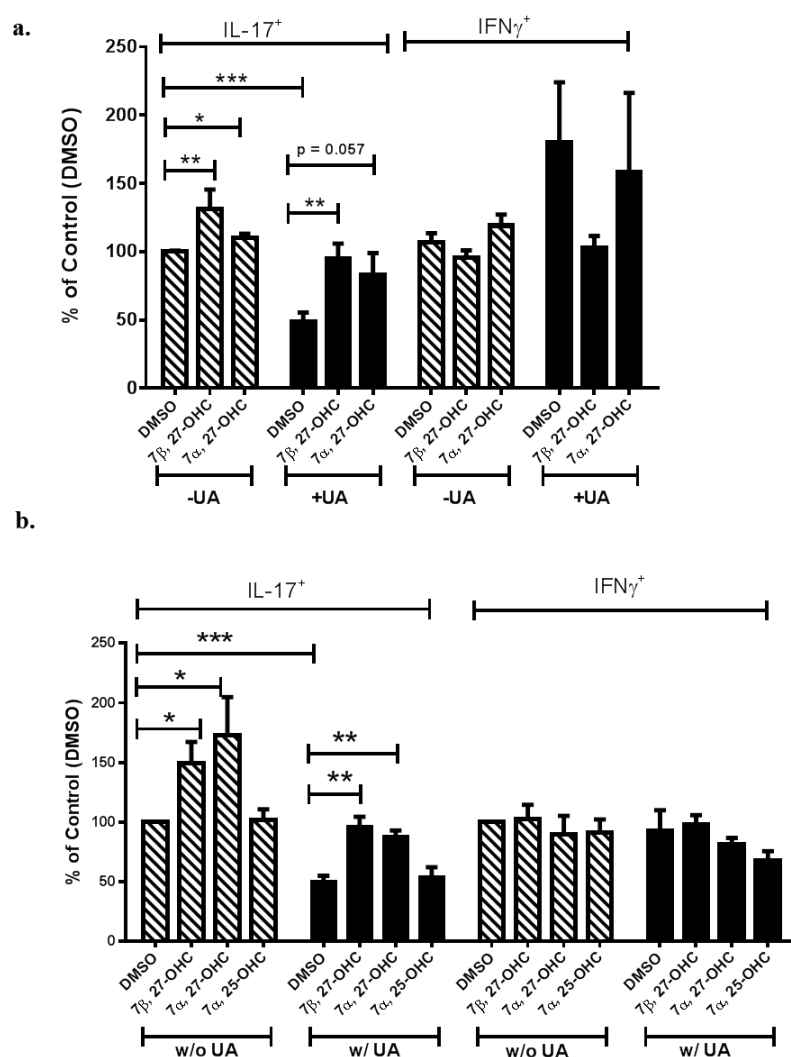


Figure S5. Dose titration of 7 β , 27-OHC in enhancing IL-17 production by mouse naïve CD4 T cells activated under Th17 differentiation condition *in vitro*. Flow cytometry analysis of intracellular staining for IL-17A and IFN γ in purified naïve CD4⁺ T cells from B6 mice activated under Th17 polarizing condition for 6 days. Titrated concentrations of 7 β / α , 27-OHC (1:3 dilution starting at 6 μ M) or DMSO were added at the beginning of the culture. % values of IL-17⁺ or IFN γ ⁺ CD4⁺ cells were used to calculate EC₅₀ with Prism.

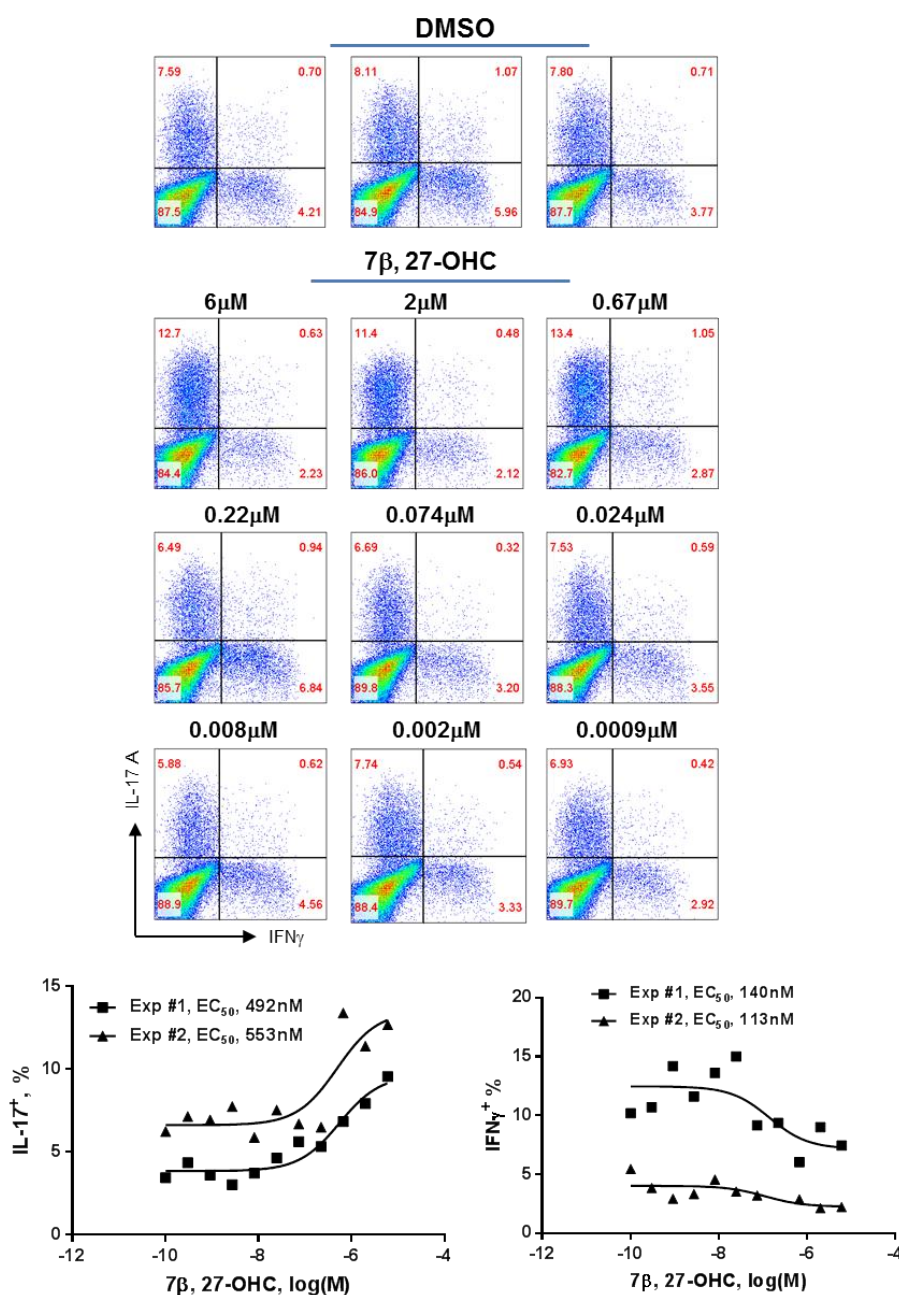


Figure S6. Dose titration of 7 β , 27-OHC in reversing inhibitory effect of ursolic acid in blocking IL-17 production by mouse CD4 T cells *in vitro*. Flow cytometry analysis of intracellular staining for IL-17A and IFN γ in purified total CD4 $^{+}$ T cells from B6 mice activated under Th17 polarizing condition for 3 days. DMSO (100% of response) or 1 μ M UA (0% of Response) was added 2 h before cell stimulation. Titrated concentrations of 7 β / α , 27-OHC (1:2 dilution) were added at the beginning of the culture. “% of Response” was then calculated based on % of IL-17 $^{+}$ CD4 $^{+}$ cells and used to calculate EC $_{50}$ with Prism.

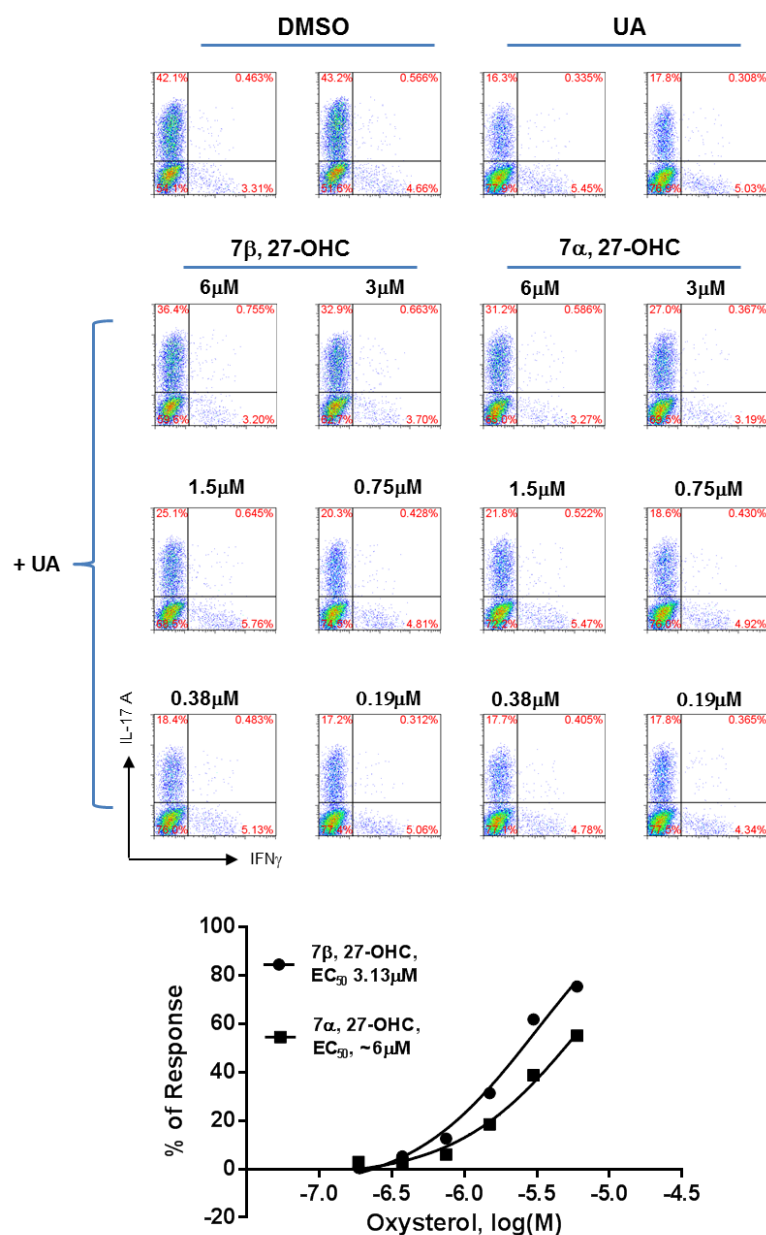


Figure S7. 7-keto, 27-OHC is less efficacious than 7 β / α , 27-OHC in enhancing IL-17 production by mouse (a) and human (b) CD4 T cells *in vitro*. Flow cytometry analysis of intracellular staining for IL-17A and IFN γ in purified naïve CD4 T cells, activated under Th17 polarizing condition for 6 days (a, mouse), or 11 days (b, human). DMSO or 1 μ M UA was added 2 h before cell stimulation. Different oxysterols (6 μ M for mouse cells; 0.3 μ M for human cells) were added at the beginning of the culture. The lower concentrations of oxysterols were used for human cells because oxysterols showed higher cytotoxicity for human T cells than mouse T cells in culture.

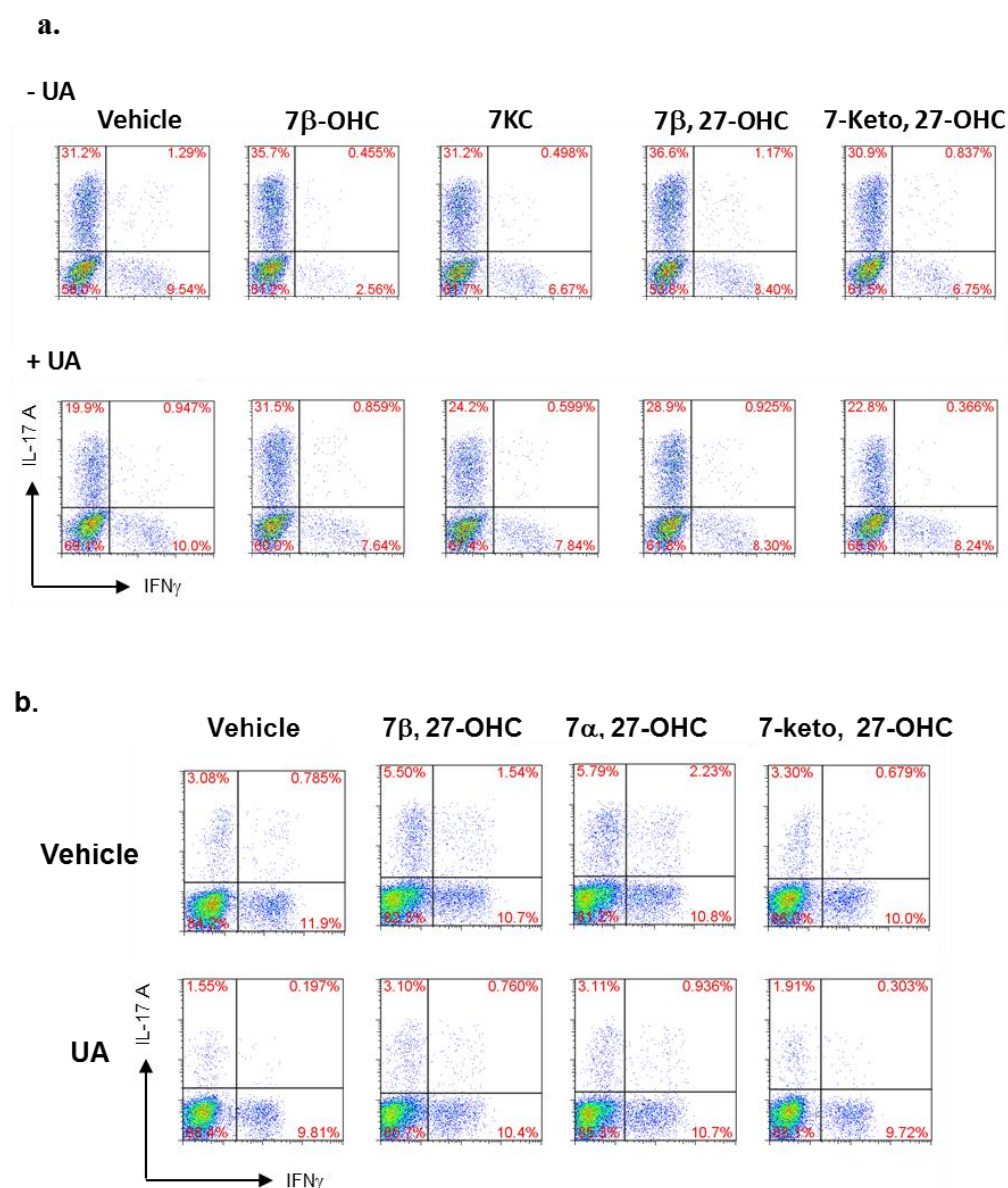


Figure S8. Key pathways based on past literatures in production of 27-hydroxylated oxysterols. Mitochondrial sterol 27-hydroxylase (CYP27A1) is a key enzyme in forming all 27-OHCs. CYP7A1 and CYP7B1 are involved in 7 α -hydroxylation of cholesterol or 27-OHC respectively. Enzyme-independent autooxidation was believed to be responsible for formation of 7 β -OHC and 7KC, and to a lesser degree maybe 7 α -OHC as well.

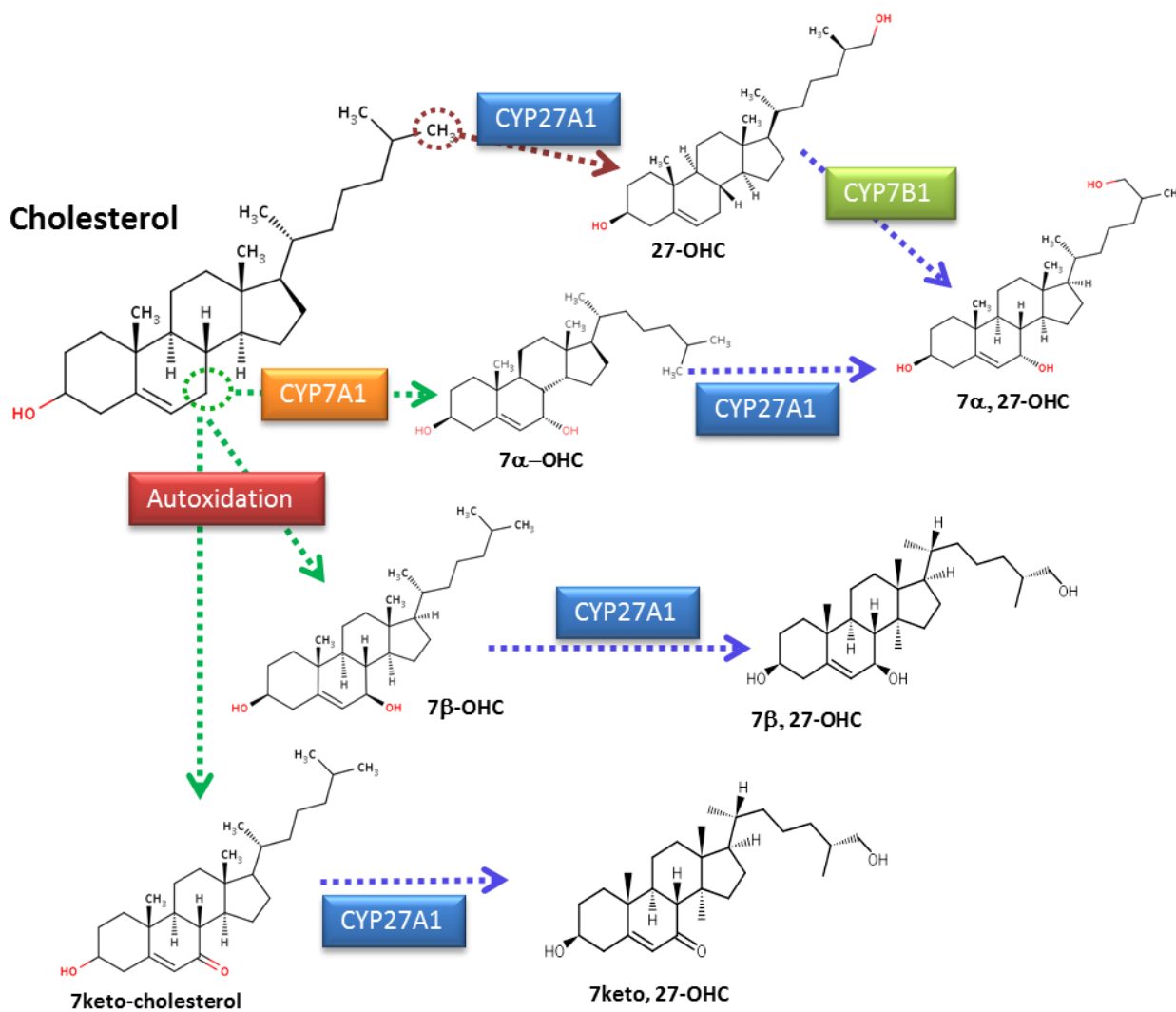


Figure S9. Representative extracted MRM ion chromatograms of selected oxysterols from a WT (in red) mouse spleen vs. a Cyp27a1 KO (in blue) mouse spleen. Raw data were shown without normalization to account for spleen weights (roughly similar) and internal standard (d6-7 α , 25-OHC, d6-27-OHC, d7-7 α -OHC, and d7-7 β -OHC) intensities. There was no apparent retention time shift between the two sample runs based on visual inspection of the internal standard peaks. Results clearly showed differences in the levels of these oxysterols between WT and Cyp27a1 KO spleen samples.

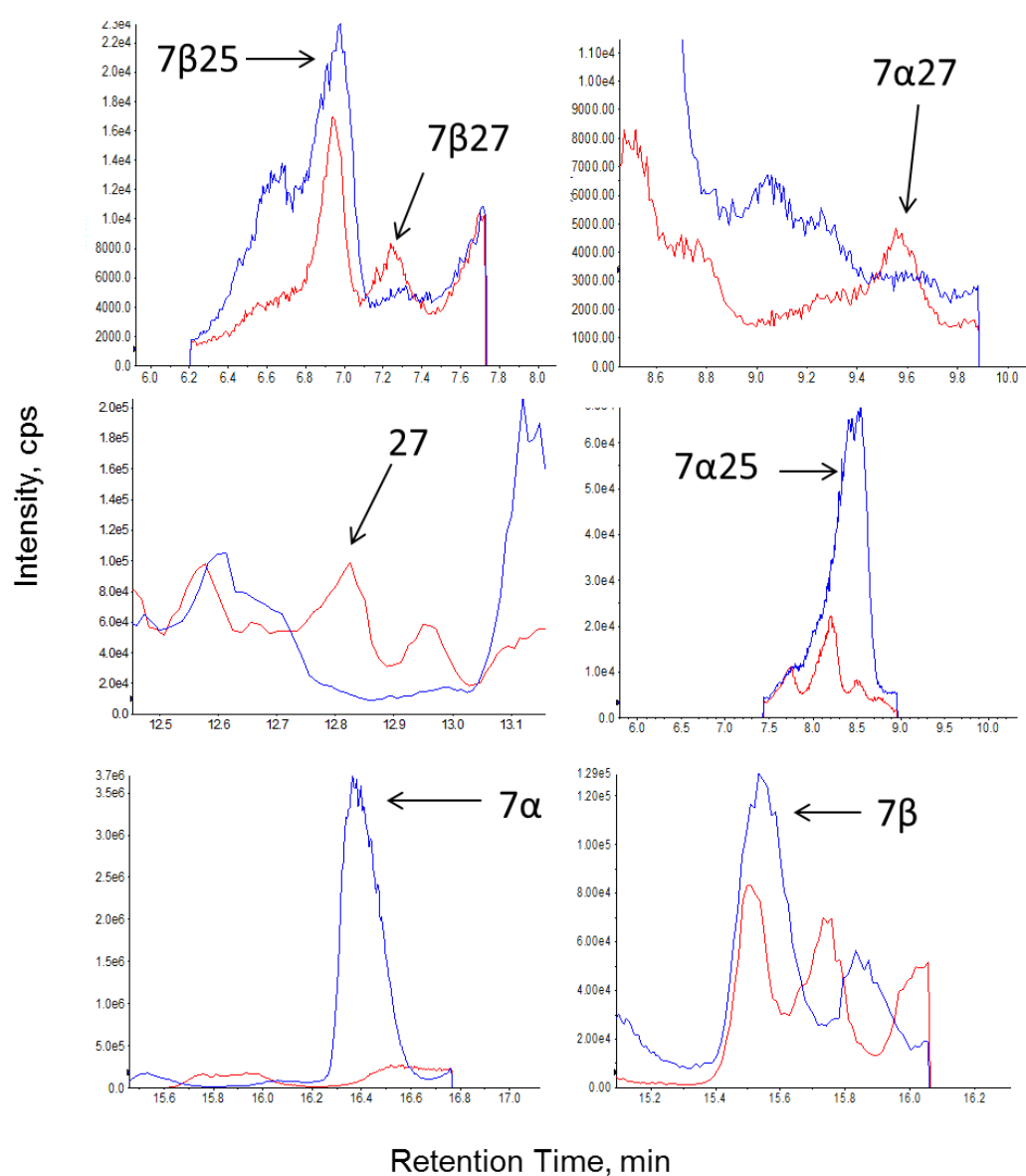
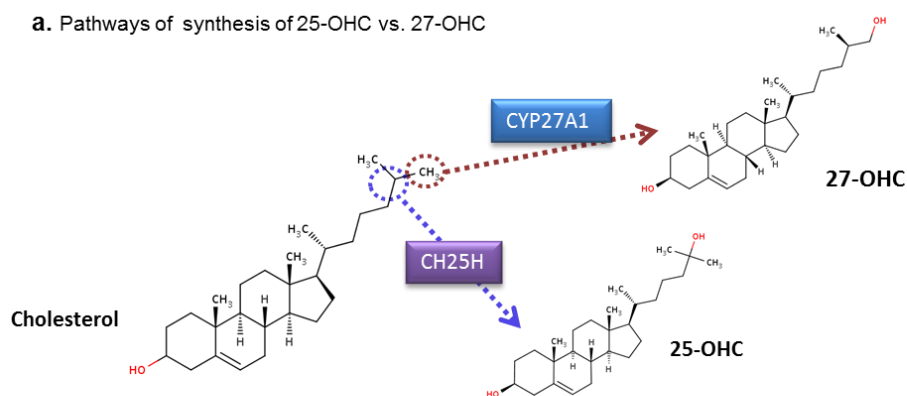
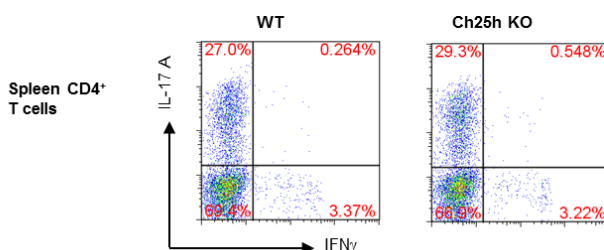


Figure S10. 25-OHCs are unlikely ROR γ t agonists in mice. **a.** Illustrated are underlying mechanisms for the production of 27- or 25-OHCs. **b.** Flow cytometry analysis of intracellular staining for IL-17A and IFN γ in gated CD4 $^{+}$ T cells from WT and Ch25h KO mouse splenocytes activated and expanded under Th17 polarizing condition for 2 days. **c.** Increased levels of 7, 25-OHCs in spleen (n=5) of Cyp27a1 KO mice. The amount of both dihydroxycholesterols was calculated compared to the spiked-in internal control, i.e. d6-7 α , 25-OHC (40ng/spleen). Statistics by Student's t-test, two-tailed, unpaired, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.



b. Normal IL-17 $^{+}$ CD4 $^{+}$ T cells in Ch25h KO mice



c. Increased levels of 7, 25-OHCs in IL-17 $^{+}$ production defective CYP27A1 KO mice

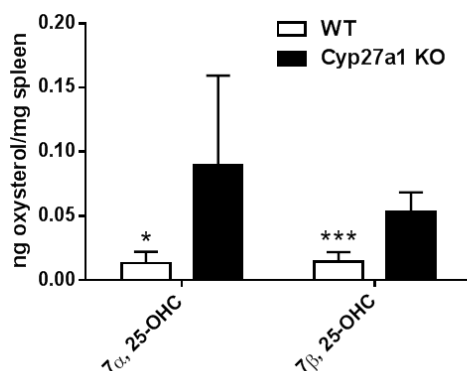


Figure S11. Defective IL-17 producing cells in ROR γ t KO mice. Flow cytometry intracellular staining analysis for IL-17A and IFN γ of spleen CD4 $^{+}$ and $\gamma\delta^{+}$ T cells from ROR γ t KO vs. WT mice. Total splenocytes were activated under Th17 polarizing condition for 3 days. RT-PCR analysis were performed on selected IL-17 pathway genes from mRNA samples (n=3). Statistics by Student's t-test, two-tailed, unpaired, * p<0.05, ** p<0.01, and ***p<0.001.

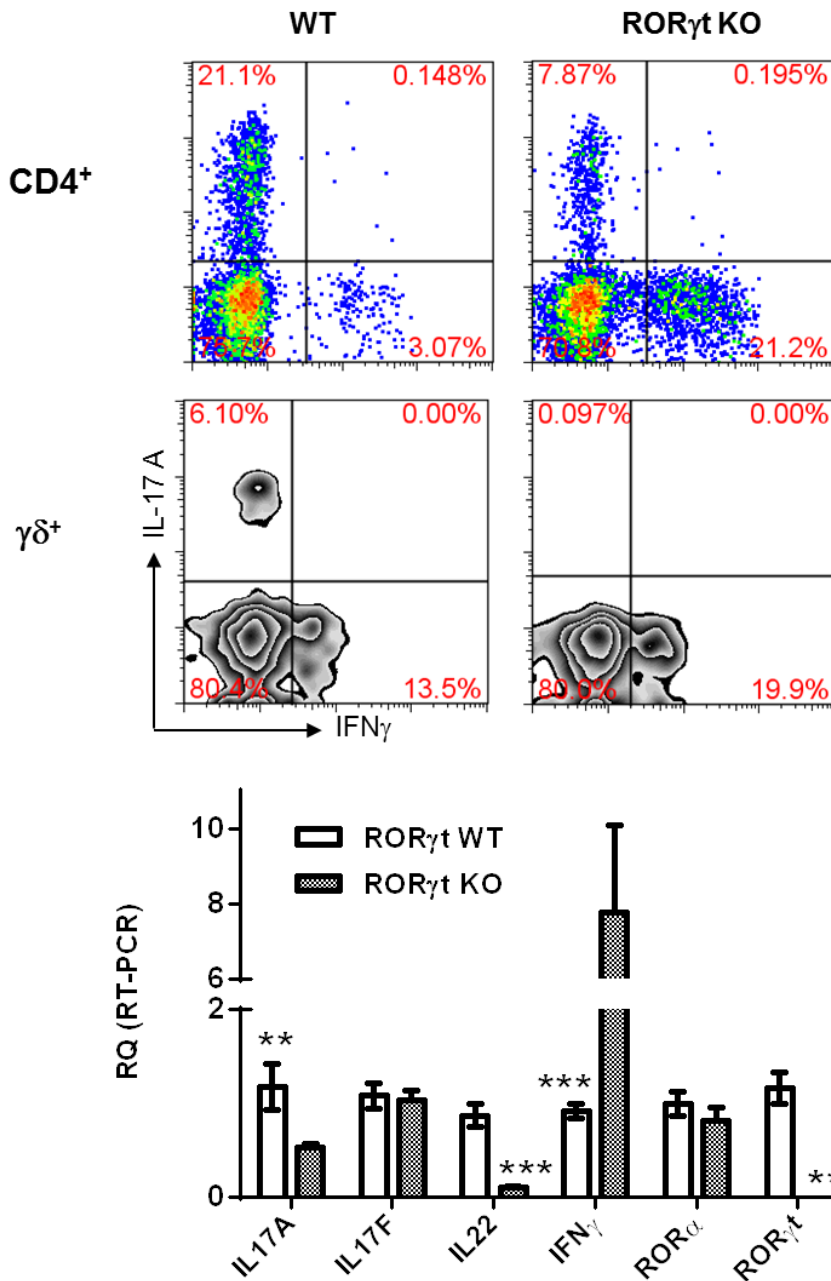


Figure S12. CYP27A1 is required for activity of 7β -OHC and 7α -OHC in driving IL-17 production by $CD4^+$ T cells *in vitro*. **a**, Effect of 7β -OHC and 7α -OHC on IL-17 production by mouse Th17 cell *in vitro*. Flow cytometry intracellular staining analysis for IL-17A and IFN γ in purified total $CD4^+$ T cells from WT mice activated and expanded in the presence of mouse Th17 polarizing cytokines for 3 days. DMSO (vehicle) or 1 μ M UA was added 2 h before cell stimulation. Oxysterols (6 μ M) were added at the beginning of the culture. **b**, Activity of 7β -OHC and 7α -OHC enhancing IL-17 production *in vitro* depends on expression of Cyp27a1 by mouse T cells. Purified naive $CD4^+$ T cells from WT or Cyp27a1 KO mice activated and expanded in the presence of mouse Th17 polarizing cytokines for 6 days. Oxysterols (6 μ M) were added at the beginning of the culture.

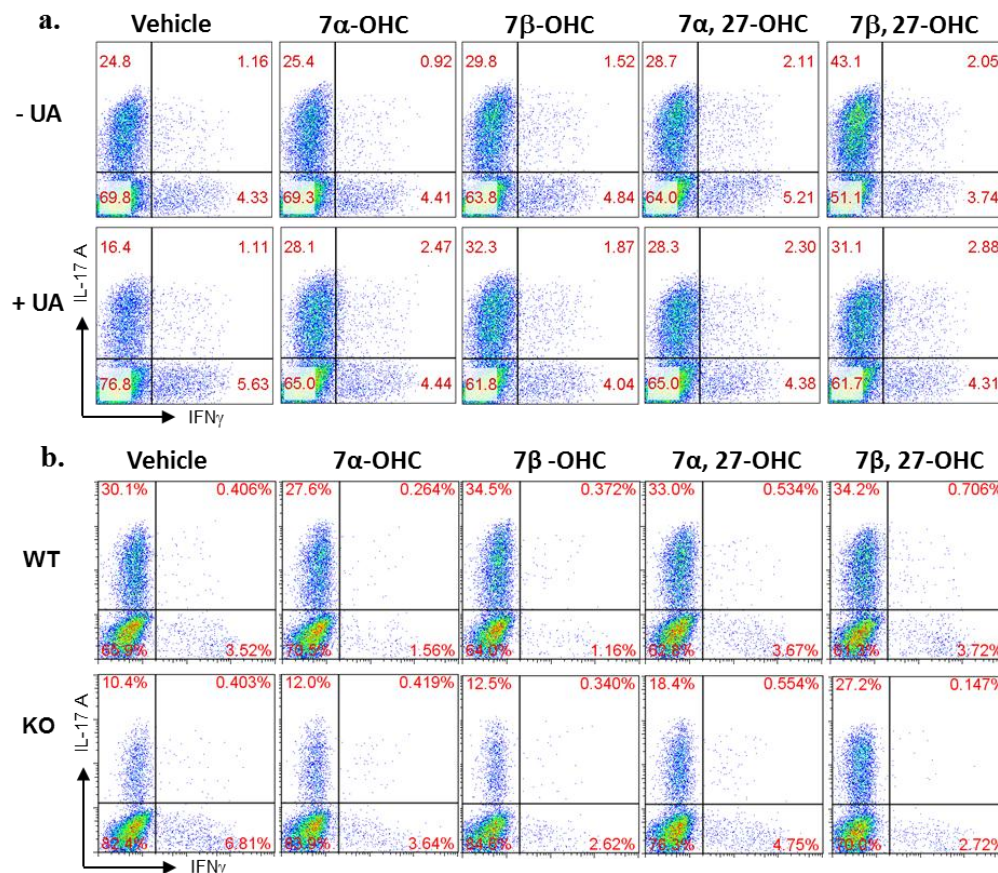


Figure S13. Expression of Cyp27a1 and Ch25h, and levels of endogenous oxysterols in mouse Th17 and Th1 cells. a. RT-PCR analysis of selected genes including Cyp27a1 and Ch25h from mouse Th17 cells and Th1 cells generated *in vitro*. Shown are average values from two runs. **b.** Levels of endogenous oxysterols in *in vitro* differentiated mouse Th17 and Th1 cells. Concentrations in nM were converted from ng/mg of wet cell pellet weight assuming 1mg equals 1 μ l in volume. Bar graphs were average \pm SEM of two LC/MS/MS runs. Statistics by Student's t-test, two-tailed, unpaired, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

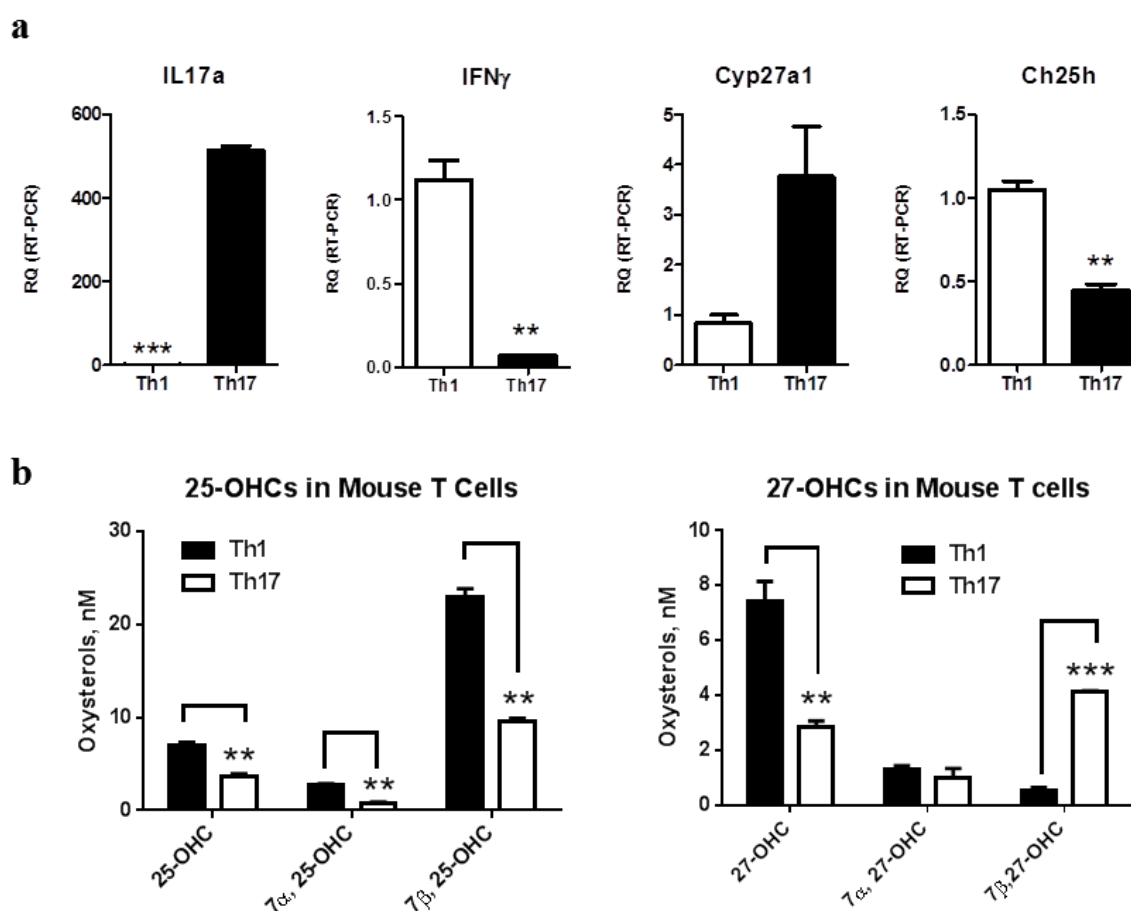
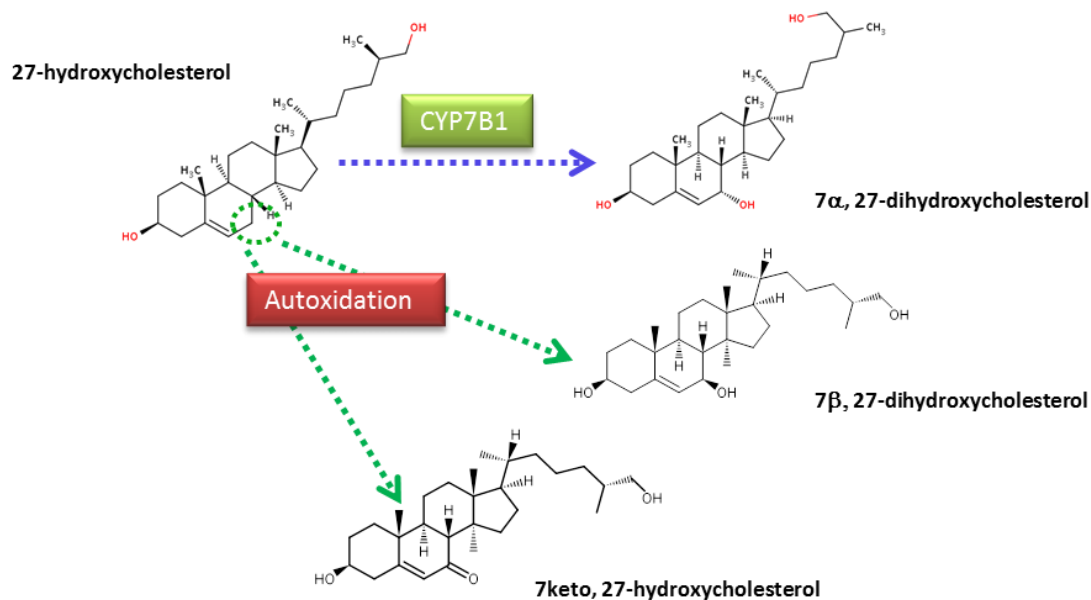


Figure S14. Minimal conversion of ^3H -27-OHC into 7, 27-OHCs by mouse Th17 or Th1 cells. **a.** Illustration of potential pathways of converting 27-OHC into 7, 27-OHCs. **b.** HPLC profile, similarly determined as described in Figure 6, of conditioned media from mouse Th17 and Th1 cell cultures spiked with ^3H -27-OHC and cultured overnight.

a. Possible pathways of conversion 27-OHC into 7, 27-OHC



a. Minimal conversion 27-OHC into 7, 27-OHC in mouse Th17 or Th1 T cells in culture

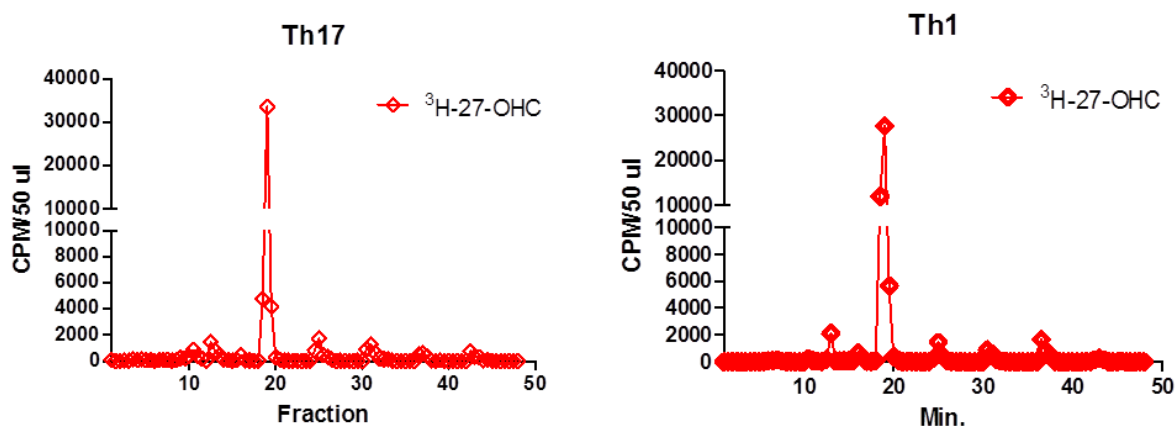


Figure S15. Representative extracted ion chromatogram of LC-MS/MS analysis of oxysterol (dihydroxyl-oxysterols are shown in green; single hydroxyl-oxysterols are shown in red) and cholesterol (shown in blue) standard solutions. Concentration of the standards: 51pg/ μ L. The oxysterols bear two E, Z isomers after the Girard P derivatization and the more dominant isomers were labeled in the chromatogram and used for quantification.

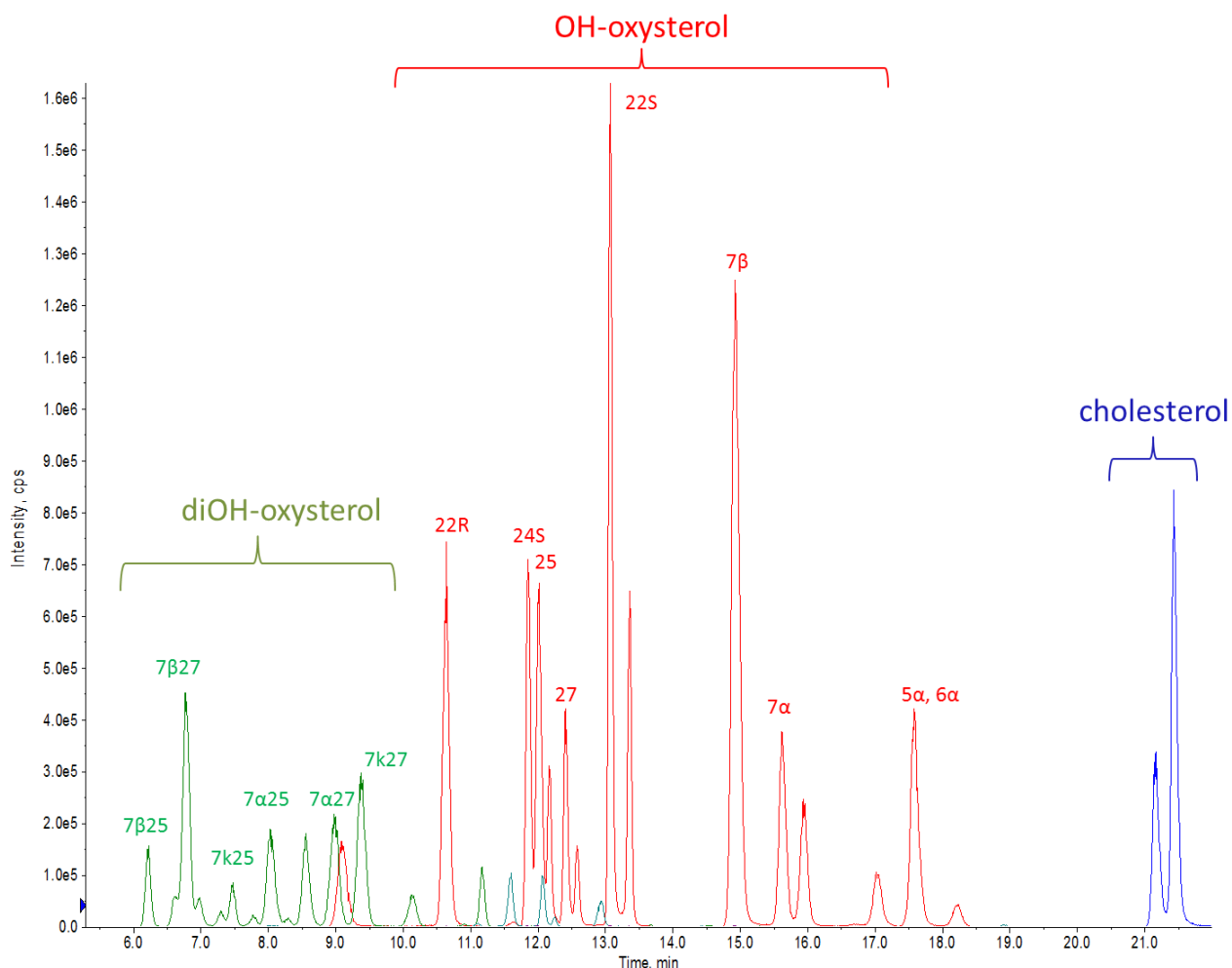


Table S1: The average EC₅₀ and efficacy of selected oxysterols in reversing inhibition by ROR γ antagonist ursolic acid in the chimeric ROR γ reporter assay (n = or > 3)

Titrated concentrations of oxysterols (starting concentration at 6 μ M, 1:3 serial dilutions) were tested in duplicated cultures of transfected HEK293T cells in the presence (0% of response) or absence (100% of response) of 1 μ M UA. EC₅₀ of oxysterols were calculated using GraphPad Prism 5. 100% Efficacy was defined as reporter activity in the absence of ursolic acid. Data shown are average and standard of derivation from at least 3 independent experiments.

Oxysterol	EC₅₀ (Ave \pm SD, nM)	Efficacy at 6μM (Ave \pm SD, %)
7-keto, 27-OHC	480 \pm 130	78 \pm 22
7 β , 27-OHC	520 \pm 120	109 \pm 8
27-OHC	650 \pm 130	121 \pm 14
22(R)-OHC	860 \pm 280	56 \pm 20
25-OHC	1,060 \pm 280	50 \pm 5
7 β , 25-OHC	1,650 \pm 590	60 \pm 8
24(S), 25-EC	1,660 \pm 380	75 \pm 14
7 β -OHC	2,190 \pm 590	39 \pm 11
7 α , 27-diOH-4-cholesten-3-one	2,530 \pm 750	42 \pm 4
7 α , 27-OHC	3,190 \pm 1,430	41 \pm 11
24(S)-OHC	4,260 \pm 1,720	82 \pm 27
7 α -OHC	5,520 \pm 2,900	26 \pm 9
7-KC	~6,000	26 \pm 8
7 α , 24(S)-OHC	>6,000	28 \pm 8
5 β , 6 β -Epoxycholestanol	>6,000	22 \pm 4
3 β , 7 β -diOH-5-cholestenoic acid	>6,000	22 \pm 13
7 α , 25-OHC	>6,000	20 \pm 2
3 β , 7 α -diOH-5-cholestenoic acid	>6,000	11 \pm 7
5 α , 6 α -Epoxycholestanol	>6,000	9 \pm 5
Cholesterol	>6,000	-2 \pm 6

Table S2: The list of compounds which were tested and found inactive up to 6 μ M in reversing inhibition by ROR γ antagonism in the chimeric ROR γ reporter assay.

Compound Name	Compound Name
Chenodeoxycholic acid	3 α ,7 α ,12 α -triOH cholestenoic acid
Estrone	Aldosterone
Cholestenone	Calcifediol
5-pregnen-3 β -ol-20-one	5 α -Cholestan-3 β -ol
Taurocholic acid	15 α -OH cholestene
Hydrocortisone	Psychosine
Lithocholic acid	5 α -Cholest-7-en-3 β -ol
Cholic acid	15 β -OH cholestene
1 α -OH vitamin D3	5 β -Cholestan-3 α -ol
Glycocholic acid	Corticosterone
Cholecalciferol	Ethynyl Androstenediol
17 α -OH-progesterone	Reichstein's substance S
1-oleoyl-sn-glycero-3-phosphocholine	21-OH-progesterone
Calcipotriol	4 β -OH cholesterol
β -Estradiol	6-Ketocholestanol
Sphingosylphosphorylcholine	Doxercalciferol
24(R)-24,25-diOH-vitamin D3	Sodium deoxycholate
25-OH cholecalciferol	Estriol
Desmosterol	1 α ,25-diOH vitamin D3
Zymosterol	17 α -OH-pregnenolone
Stigmasterol	5 α -Cholestan-3-one
Lathosterol	Progesterone
Lanosterol	7-Dehydrocholesterol
Lanostenol	5 α -Cholestane
14-Demethyl-lanosterol	<i>trans</i> -Dehydroandrosterone
Sphingosine 1-phosphate	

Table S3: sMRM parameters of oxysterol standards for LC/MS/MS method from a representative experiment. ¹ Girard reagent P used for derivatization; ² Girard reagent P derivatization on 3-oxo-4-ene oxysterols; ³ Parent ion mass in positive ion mode; ⁴ Most abundant fragment ion mass; ⁵ Retention time on chromatography; ⁶ Declustering potential; ⁷ Collision energy.

Compound name	Source	GP ¹ labeled Compound ID and abbreviation	Q1 ³	Q3 ⁴	RT ⁵ (min)	DP ⁶	CE ⁷	Internal standard used for quantification
7 α -hydroxycholesterol	Avanti Polar Lipids, 700034	3GP ² -7 α	534.4	455.4	15.61	30	45	3GP-7 α -d7
7 α -hydroxycholesterol (d7)	Avanti Polar Lipids, 700043	3GP-7 α -d7	541.4	462.5	15.52	30	45	
7 β -hydroxycholesterol	Avanti Polar Lipids, 700035	3GP-7 β	534.4	455.4	14.93	30	45	3GP-7 β -d7
7 β -hydroxycholesterol (d7)	Avanti Polar Lipids, 700044	3GP-7 β -d7	541.5	462.3	14.82	30	45	
22(S)-hydroxycholesterol	Avanti Polar Lipids, 110813	3GP-22S	534.4	455.4	13.07	30	45	3GP-22R-d7
22(R)-hydroxycholesterol	Avanti Polar Lipids, 700058	3GP-22R	534.4	455.3	10.64	30	45	3GP-22R-d7
22(R)-hydroxycholesterol (d7)	Avanti Polar Lipids, 700052	3GP-22R-d7	541.6	462.6	10.52	30	45	
24(S)-hydroxycholesterol	Avanti Polar Lipids, 700061	3GP-24S	534.5	455.4	11.85	30	45	3GP-24RS-d7
24(R/S)-hydroxycholesterol (d7)	Avanti Polar Lipids, 700018	3GP-24RS-d7	541.4	462.0	12.20	30	45	
25-hydroxycholesterol	Avanti Polar Lipids, 110816	3GP-25	534.4	455.4	12.01	30	45	3GP-25-d6
25-hydroxycholesterol (d6)	Avanti Polar Lipids, 700053	3GP-25-d6	540.4	461.4	11.94	30	45	
27-hydroxycholesterol	Avanti Polar Lipids, 110818	3GP-27	534.4	455.4	12.41	30	45	3GP-27-d6
27-hydroxycholesterol (d6)	Avanti Polar Lipids, 700059	3GP-27-d6	540.4	461.4	12.34	30	45	
7 α ,25-dihydroxycholesterol	made in-house	3GP-7 α ,25	550.4	471.4	8.03	30	45	3GP-7 α ,25-d6
7 α ,25-dihydroxycholesterol (d6)	Avanti Polar Lipids, 700078	3GP-7 α ,25-d6	556.4	477.4	7.94	30	45	
7 β ,25-dihydroxycholesterol	made in-house	3GP-7 β ,25	550.4	471.4	6.22	30	45	3GP-7 α ,25-d6
7 α ,27-dihydroxycholesterol	Avanti Polar Lipids, 110824	3GP-7 α ,27	550.4	471.4	8.97	30	45	3GP-7 α ,25-d6
7 β ,27-dihydroxycholesterol	Avanti Polar Lipids, 700025	3GP-7 β ,27	550.4	471.4	6.77	30	45	3GP-7 α ,25-d6
7-ketocholesterol	Sigma, C2394	3GP-7kC	534.4	455.4	14.93	30	45	3GP-27-d6
3 β ,25-dihydroxy-5-cholesten-7-one	made in-house	3GP-7k,25	550.4	471.4	7.47	30	45	3GP-25-d6
3 β ,27-dihydroxy-5-cholesten-7-one	Avanti Polar Lipids, 700022	3GP-7k,27	550.4	471.4	9.37	30	45	3GP-7 α ,25-d6
5 α ,6 α -epoxycholestanol	Sigma, C2773	3GP-5 α ,6 α -EPO	534.4	455.4	17.58	30	45	3GP-25-d6
5 α ,6 α -epoxycholestanol (d7)	Avanti Polar Lipids, 700047	3GP-5 α ,6 α -EPO-d7	541.5	462.6	17.44	30	45	
5 β ,6 β -epoxycholestanol	Sigma, C2648	3GP-5 β ,6 β -EPO	534.4	455.4	17.58	30	45	3GP-25-d6
24(R/S),25-epoxycholesterol	Avanti Polar Lipids, 700037	3GP-24RS,25-EPO	532.4	453.4	12.94	30	45	3GP-24RS,25-EPO-d6
24(R/S),25-epoxycholesterol (d6)	Avanti Polar Lipids, 700048	3GP-24RS,25-EPO-d6	538.4	459.2	12.86	30	45	

Table S4: Levels of oxysterols in total spleen of Cyp27a1 KO and WT controls. Oxysterols were quantitated with a stable isotope dilution technique via a LC/MS/MS method. Standard curves (representatives shown below) were generated by mixing the standard stocks with the dilution solution (cholesterol 0.15 mg/ml, BSA 0.5 mg/ml, 1 mg/ml $\text{Na}_2\text{S}_2\text{O}_5$ in 30% water, 70% EtOH) at 7 final concentrations (0.03, 0.09, 0.27, 0.81, 2.43, 7.29, 21.87ng). 10 μL of the deuterated internal standard stock (1ng/ μL) was spiked to each standard curve samples and spleen samples. The derivatization and following extraction steps for both standard curve and spleen samples were carried out in parallel. For this specific run, there was apparent and unknown source of matrix interference with 7α , 27-OHC*, which was not differentiable with our current method. Therefore, we believed the high levels of 7α , 27-OHC observed in Cyp27a1 KO samples should be considered as background.

Oxysterol	Concentrations in Whole Mouse Spleen (Ave \pm SD, nM, n=4)	
	WT	Cyp27a1 KO
7α -OHC	779.1 \pm 1364.7	42609.4 \pm 5006.3
7β -OHC	159.0 \pm 156.4	2631.1 \pm 2632.9
25-OHC	22.8 \pm 20.5	72.9 \pm 12.3
7α , 25-OHC	5.3 \pm 2.8	308.5 \pm 48.3
7β , 25-OHC	1.3 \pm 0.8	20.3 \pm 9.0
27-OHC	76.8 \pm 26.2	0.3 \pm 0.5
7α , 27-OHC	135.4 \pm 13.6*	99.5 \pm 16.2*
7β , 27-OHC	3.1 \pm 2.1	0.9 \pm 1.9

